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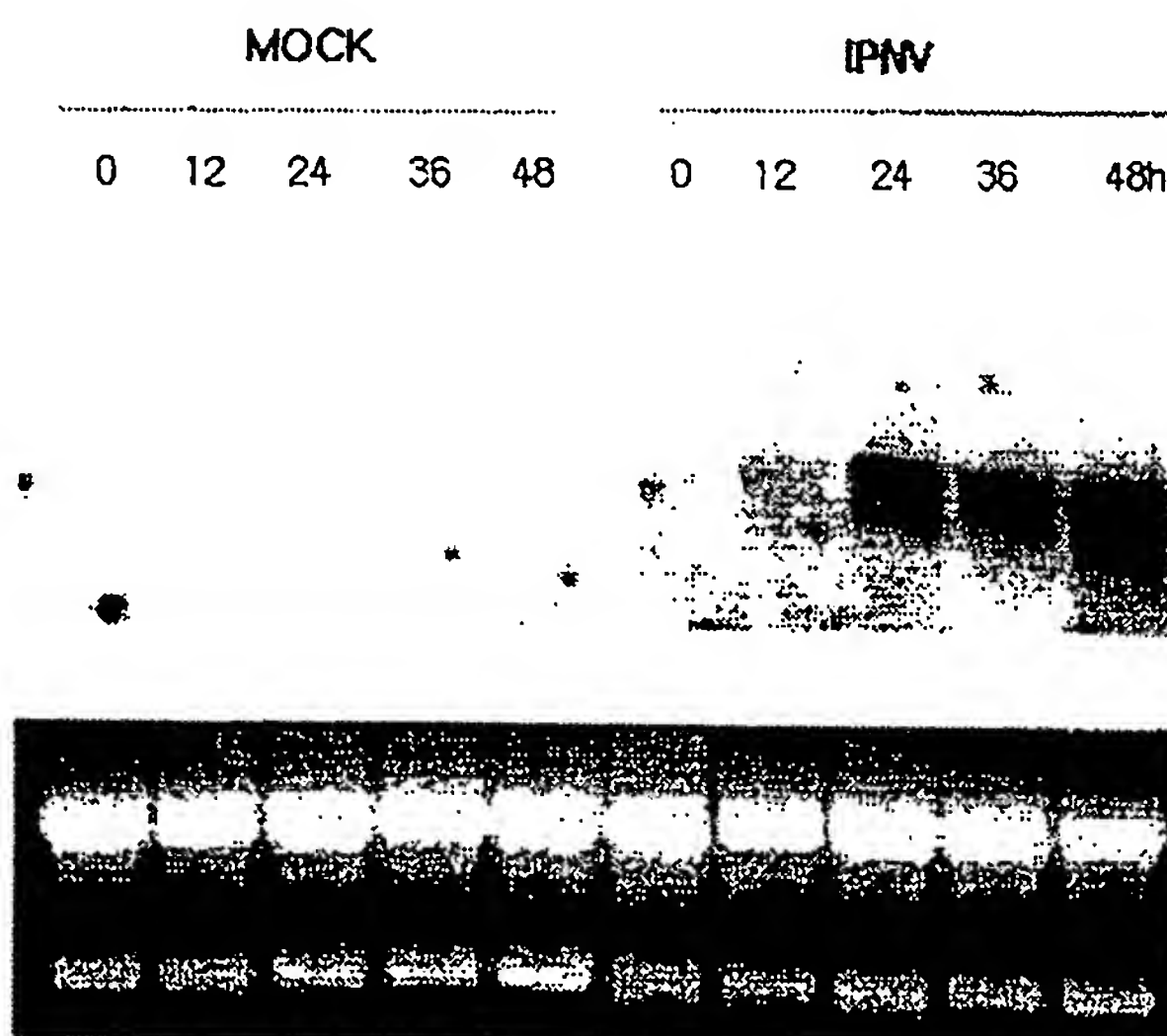
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(54) Title: NOVEL STRESS PROTEIN WITH CHAPERONE ACTIVITY



(57) Abstract: The present invention is directed to protein NSP having chaperone activity whose expression level increases in cells of an animal subjected to external stresses, and to a process for producing said protein NSP. Additionally, the present invention is directed to nucleic acid molecules encoding such protein NSP, recombinant vectors comprising said nucleic acid molecules, host cells transformed with such recombinant vectors, antibodies against such protein NSP, and pharmaceutical or diagnostic compositions and vaccines containing said protein NSP, vectors or antibodies for preventing or treating or diagnosing infectious diseases of animals. Further, the present invention is directed to inhibitors of activity or expression of such protein NSP, and to a method for screening said inhibitors. Furthermore, the present invention is directed to a protein array including such protein NSP and a solid support.



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## NOVEL STRESS PROTEIN WITH CHAPERONE ACTIVITY

**Technical Field**

5           The present invention relates to the protein NSP having chaperone activity whose expression level increases in cells of an animal subjected to external stresses, and to a process for producing the protein NSP. Also, the present invention relates to nucleic acid molecules encoding the protein NSP, recombinant vectors comprising the nucleic acid molecules, host cells transformed with such recombinant vectors, antibodies against the protein NSP, and  
10   pharmaceutical or diagnostic compositions and vaccines comprising the protein NSP, vectors or antibodies for preventing or treating or diagnosing infectious diseases of animals. Further, the present invention relates to inhibitors of activity or expression of the protein NSP, and to a method for screening said inhibitors. In addition, the present invention relates to a protein array including the protein NSP and a solid support.

**Background Art**

15           Heat shock proteins or stress proteins are induced by various stressful stimuli such as heat shock, malnutrition, oxygen radical, metabolism deficiency, heavy-metal and virus  
20   infection and functions to protect cells from damage occurred by external stimuli by acting as a molecular chaperone. The stress proteins exist in both procaryotes and eukaryotes and perform various biological functions. These proteins are known that they concern with several functions such as protein folding, protein targeting to membranes or protein renaturation or degradation after given a stress thereto and regulation of protein-protein

interactions, and form a family of polypeptide-binding proteins (Ellis et al., *Annu. Rev. Biochem.*, 60, 321-347 (1991); Georgopoulos et al., Cold spring Harbor Laboratory, Cold Spring Harbor, NY, 209-250 (1994); and Hendrick et al., *Annu. Rev. Biochem.*, 62, 349-384 (1993)).

5           The stress proteins are divided into major three families of Hsp60, Hsp70 and Hsp90 according to their molecular weights (Hemmingsen et al., *Trends Biochem. Sci.*, 14:339-342, 1989; and Hartl et al., *Annu. Rev. Biochem.*, 62:349-384, 1993). Hsp60 and Hsp70, having a size of about 60 kDa and 70 kDa, respectively, exist in cytoplasm in great amount and perform chaperone activities. It is known that Hsp70 is induced of its  
10       expression when virus is penetrated into the cell as well as a temperature rise. For related literatures to above fact, Shopping et al. have reported that paramyxovirus 5 and rotavirus cause an increase in synthesis of GRP78 in cells (Shoppin et al., *Proc. Natl. Acad. Sci. U.S.A.*, 75:6120-6124, 1978, Taylor et al., *J. Virol.*, 72:9865-9872, 1998), and also Rubi et al. have reported that vaccinia virus induces the expression of Hsp70 (Ruby et al., *J. Virol.*,  
15       68:4685-4689, 1994).

Hsp90 has chaperone activity like HSP60 and Hsp70, and also regulates the activities of the proteins as it exists in a cytoplasm and combines with a steroid hormone receptor etc. In addition, Hsp90 is preserved in bacteria, yeast and mammal in a high rate, similarly to HSP60 and Hsp70 etc. (Craig, et al., *Trends Biochem. Sci.*, 16, 135-139 (1993)).  
20       As compared with other stress proteins, Hsp90 is shown to stabilize various kinds of target proteins, especially in inert state or unassembled state (Craig E. A., *Annu. Rev. Biochem.*, 60, 321-347 (1991)).

As mentioned above, the expression of stress proteins is induced by the various stimuli, one of which is the virus infection, and in certain cases, the stress proteins may be



found in combination with the virus. For example, the stress proteins are found as  
complexes with adenovirus proteins (Macejak, et al., J. Virology, 180, 120-125 (1991)),  
vaccinia viruses proteins (Jindal, et al., J. Virology, 66, 5357-5361 (1992)), vesicular  
stomatitis virus proteins (Hammond, et al., Science, 266, 456-459 (1994)), polyoma virus  
5 (Lindquist, S., Annu. Rev. Biochem., 55, 1151-1191 (1986); Lindquist, et al., Annu. Rev.  
Genet., 22-631-677 (1988)), SV40 (welch, W. J., CSH Laboratory Press, NY, 223-278  
(1990)), sindbis virus (Mulvey, et al., J. Virology, 69, 1621-1627 (1995)), rabies virus  
(Sagara, et al., Virology, 190, 845-848 (1992)) and canine distemper virus (Oglesbee, et al.,  
J. General Virology, 71, 1585-1590 (1990)). It is speculated that stress proteins functions to  
10 act as molecular chaperones facilitating the folding, translocation and assembly of viral  
peptides newly formed during virus infection.

Evidences, which the stress protein is a major antigen against lots of pathogens,  
have been raised recently. Hsp60 family proteins, also called as GroEL-related proteins,  
have been reported as the major antigen against various classes of bacterial pathogens  
15 including *Mycobacterium leprae* and *Mycobacterium tuberculosis* (D. Young et al., Proc.  
Natl. Acad. Sci. USA, 85, 4267-4270 (1988)), *Legionella pneumophila* (B. B Plikaytis et al.,  
J. Clin. Microbiol., 25, 2080-2084 (1987)), *Borrelia burgdorferi* (B. J. Luft et al., J.  
Immunol., 146, 2776-2782 (1981)) and *Chlamydia trachomatis* (E. A. Wagar et al., J. Infect.  
Dis., 162, 922-927 (1990)). It is also observed that, in case of some bacterial and parasitic  
20 infections, antibodies against Hsp70 family members or DnaK-related proteins are generated  
(Young et al., Supra; Luft et al., supra; D.M. Engman et al., J. Immunol., 144, 3987-3991  
(1990); N. M. Rothstein et al., Molec. Biochem. Parasitol., 33, 229-235 (1989); V.  
Nussenzweig and R. S. Nussenzweig, Adv. Immunol., 45, 283-334 (1989)).

Stress proteins are known as inducing strong B- and T- cell reactions, and therefore,

immunoreactions against stress proteins are regarded as to be one of the mechanisms which protect cells from infection caused by virus, etc. The fact that antibodies against stress proteins of microorganisms or T-cells, reacting with them, show counteractive and protective activities also supports such hypothesis (A. Noll et al., Infect. Immun., 62, 2784-2791 (1994); S. L. Danilition et al., Infect. Immun., 58, 189-196 (1990)).

As stated above, stress proteins have been watched as a component of a vaccine and therapeutic agent due to their immunological properties, and, for this reason, the finding of a novel stress protein which can provide a method for diagnosis, treatment and prevention of diseases can be very useful for mankind.

### Disclosure of the Invention

Accordingly, the present invention provides a protein NSP with chaperone activity which is expressed in an increased level in animal cells stimulated by external stresses. More particularly, the present invention provides a novel stress protein which shows an increase in the expression levels in cells of animals which are given an external stress, such as pathogenic infection, heat shock, malnutrition, toxic metal, oxygen radical, metabolism collapse, analogues, derivatives and homologues thereof, and fragments having at least one immunological epitope. Also, the present invention provides proteins represented by SEQ ID NO 1.

The present invention provides a method for producing the novel stress protein with chaperone activity which show increases in the expression levels in cells of an animal which is given to an external stress.

Also, the present invention provides a method for producing substantially pure protein NSP, which comprises steps of (a) inserting DNA sequence encoding the protein into

a vector having at least one of expression control sequences which is operatively linked to the DNA sequence and controls expression of the DNA sequence to form a recombinant expression vector, (b) transforming the recombinant expression vector into a host cell, (c) culturing the transformed cell in suitable media and conditions to express the DNA sequence, and (d) recovering substantially pure protein NSP from the culture.

The present invention provides DNA sequences encoding protein NSP and polynucleotide sequences hybridizing thereto. Particularly, the present invention provides DNA sequences encoding protein NSP and represented by SEQ ID NO: 2, and polynucleotide sequences hybridizing thereto.

The present invention provides recombinant expression vectors comprising the nucleotide sequences and fragments thereof, particularly the nucleotide sequence encoding protein NSP and represented by SEQ ID NO: 2 and fragments thereof.

Also, the present invention provides host cells transformed or transfected by the recombinant expression vectors, particularly, the nucleotide sequence encoding protein NSP and represented by SEQ ID NO: 2 (hereinafter referred as "NSP DNA sequence" and fragments thereof.

The present invention provides antibody recognizing the protein NSP and hybridoma producing the antibody.

The present invention provides inhibitors of the protein NSP. Particularly, the present invention provides inhibitors which can inhibit activity or expression of the protein NSP.

The present invention provides a pharmaceutical composition for preventing or treating infectious diseases of animals infected by pathogenic organisms comprising a therapeutically or prophylactically effective amount of an inhibitor and/or antibody against

the protein NSP and a pharmaceutically acceptable carrier.

Also, the present invention provides a pharmaceutical composition for preventing or treating infectious diseases of animals infected by pathogenic organisms characterized by comprising the protein NSP, DNA sequence encoding the protein or a nucleotide fragment thereof, or a recombinant vector containing the foregoing in an amount sufficient to induce immunity.

Also, the present invention provides a vaccine for preventing or treating infectious diseases of animals infected by pathogenic organisms comprising an immunologically effective amount of the protein NSP.

Also, the present invention provides a DNA vaccine for preventing or treating infectious diseases of animals infected by pathogenic organisms comprising DNA sequence encoding the protein NSP or a nucleotide fragment thereof in an amount sufficient to induce immunity.

Also, the present invention provides a composition for diagnosis of diseases accompanying with an increased expression of the protein NSP comprising a nucleic acid molecule encoding the protein NSP.

Also, the present invention provides a method for diagnosing diseases accompanying with an increased expression of the protein NSP comprising (a) contacting a biological sample of animal with an antibody against the protein NSP and (b) detecting the presence of the conjugate of the protein NSP in the biological sample with the antibody.

Also, the present invention provides a method for diagnosing diseases accompanying with an increased expression of the protein NSP comprising (a) reacting a biological sample of animal with a polynucleotide encoding the protein NSP and (b) detecting a nucleic acid molecule in the sample which hybridizes with a polynucleotide

encoding the protein.

Also, the present invention provides a method for diagnosing diseases accompanying with an increased expression of the protein NSP comprising (a) assaying an expression level of a polynucleotide gene encoding the protein NSP in a biological sample of animals and (b) comparing the assayed expression level of the gene with a standard gene expression level of the protein.

Also, the present invention provides a method for screening an antagonist against expression of the protein NSP gene comprising (a) assaying an expression level of polynucleotide gene encoding the protein NSP in animal cells infected with pathogens, (b) assaying the expression level of the polynucleotide gene in the same normal animal cells treated with the substance to be tested and then infected with the same pathogens, and (c) comparing the expression level of the protein NSP gene assayed in (a) with the expression level of the protein NSP gene assayed in (b) and determining the substance as an inhibitor where the expression level of the protein NSP gene assayed in (a) is higher than the expression level of the protein NSP gene assayed in (b).

Also, the present invention provides a protein array comprising the protein NSP or its antibody and a solid support.

Also, the present invention provides an antisense oligonucleotide which inhibits expression of the protein NSP.

Also, the present invention provides a method for screening a compound which inhibits expression of the protein NSP coding gene, comprising (a) contacting a host cell comprising the the protein NSP coding gene or expressing the gene with any compounds and (b) selecting a compound capable of suppressing expression of the protein NSP coding gene.

Also, the present invention provides a method for screening a compound which



inhibits activity of the protein NSP, comprising (a) contacting the protein NSP with any compounds and (b) selecting a compound capable of suppressing activity of the protein NSP.

Also, the present invention provides a method for folding or renaturation of a protein comprising treating the protein with the protein NSP.

5 Also, the present invention provides a method for folding or renaturation of a protein comprising treating the protein with the protein NSP which specifically binds to an antibody against the protein NSP.

Additional advantages, objects, and features of the invention will be set forth in part in the description which follows and in part will become apparent to those having ordinary  
10 skill in the art upon examination of the following or may be learned from practice of the invention.

### **BRIEF DESCRIPTION OF THE DRAWINGS**

15 The accompanying drawings, which are included to provide a further understanding of the invention and are incorporated in and constitute a part of this application, illustrate embodiment(s) of the invention and together with the description serve to explain the principle of the invention. In the drawings;

20 FIG. 1 is photographs of protein staining and western blotting, confirming separation of the protein NSP according to the present invention in CHSE-214 cell line infected with IHNV virus (A: a photograph of developing immunoprecipitated protein NSP(lane 1) and total protein of the cell lysate (lane 2) on SDS-polyacrylamide gel, followed by being stained with a coomassie blue solution, B: a photograph of western blotting, using monoclonal antibody BB10 against the protein NSP according to the present invention)

FIG. 2 illustrates the kinds and locations of motifs and domains, which are identified by analyzing the amino acid sequence of the protein NSP according to the present invention;

FIG. 3 illustrates the result of an experiment determining sequence similarities between the amino acid sequence of the protein NSP according to the present invention and sequences of other prokaryotes oligopeptide-binding protein;

FIG. 4 is a schematic view of constructing pET29-NSP vector by inserting cDNA of the protein NSP according to the present invention into NcoI-XhoI restriction site of pET29b;

FIG. 5 illustrates the results of experiments confirming products obtained by cloning the protein NSP gene according to the present invention into pET29b vector and transforming *E. coli* with the resulting recombinant vector, followed by mass-purification (a: the result of developing the protein NSP according to the present invention on SDS-polyacrylamide gel, followed by staining with coomassie blue, b: the result of western blotting, using monoclonal antibody BB10 against the protein NSP according to the present invention, in which Lane 1 is the recombinant protein NSP according to the present invention, which is expressed in *E. coli* and Lane 2 is the protein NSP of the present invention, which is separated from CHSE-214 cells by using of immunoprecipitation);

FIG. 6 illustrates the result of northern blotting, confirming the expression of the NSP gene according to the present invention in CHSE-214 cells infected with virus, in which MOCK represents cells not infected with virus and IPNV represents cells infected with virus;

FIG. 7a illustrates the result of measuring  $\alpha$ -glucosidase activity after treatment with 1  $\mu$ M protein NSP, 5  $\mu$ M DnaK, 20  $\mu$ M BSA, to confirm the influence of the protein NSP of the present invention on folding of  $\alpha$ -glucosidase (○ : control; ■: treated

with 1  $\mu$  M protein NSP,  $\square$ : treated with 5  $\mu$  M DnaK,  $\bullet$  : treated with 20  $\mu$  M BSA);

FIG. 7b a graph showing the influence of the protein NSP according to the present invention on agglomeration of alcohol dehydrogenase, in which agglomeration of alcohol dehydrogenase is measured after treatment with 0.5mM and 1.0mM of the protein NSP,;

5 FIG. 8 is a graph showing the influence of ascorbic acid on the expression of the protein NSP according to the present invention, in which CHSE-214 cells are treated with 1  $\mu$  M, 3  $\mu$  M, and 5 $\mu$ M of ascorbic acid and the protein NSP is measured by Western blotting;

10 FIG. 9 is a graph showing the influence of L-NAME, an inhibitor of NOS, on the expression of the protein NSP according to the present invention, in which CHSE-214 cells are treated with 0.5mM, 1mM, 3 mM of L-NAME and the protein NSP is measured by Western blotting;

15 FIG. 10 is a graph showing the influence of antisense oligomer of the protein NSP according to the present invention on the expression of the protein NSP and growth of IPNV and IHNV, in which CHSE-214 cells are treated with 10  $\mu$  M and 20  $\mu$  M of antisense oligomer, and the protein NSP is measured by Western blotting and IPNV and IHNV are measured by TCID50; and

20 FIG. 11 is a graph showing the protein NSP levels in sera of normal person and patients with rectal cancer and breast cancer with sandwich ELISA, to confirm the increase of the protein NSP according to the present invention in serum of a patient with cancer.

### **Best Mode for Carrying Out the Invention**

Now, the present invention will be explained in detail.

In one aspect, the present invention provides the protein NSP which shows increases in its expression level in the bodies of animals, particularly fishes subjected to external stresses such as pathogenic infection, increase of temperature, toxication by heavy metals, dextrose deficiency, etc. and shows chaperone activity. Particularly, the protein NSP according to the present invention includes protein denoted as SEQ ID NO: 1 or analogues, derivatives, homologues and fragments containing at least one immunological epitope which show increases in their expression levels in cells of animals which are given of external stresses and shows chaperone activity, In another aspect, the present invention provides the protein NSP which has chaperone activity and shows an increase in the expression level in the bodies of animals, particularly fishes when external stresses such as pathogenic infection, increase of temperature, toxication by heavy metals, dextrose deficiency, etc. are applied, and specifically bind to antibodies against the protein NSP.

The term "pathogen", as used herein, refers to all organisms directly or indirectly doing harm to any host, which particularly include virus, bacteria and parasites and the like.

In one embodiment, the viral pathogens include, but are not limited to, rhabdovirus including infectious hematopoietic necrosis virus (IHNV), viral hemorrhagic septicemia virus (VHSV), and spring viremia of carp virus (SVCV); birnavirus including infectious pancreatic necrosis virus (IPNV); marine herpesvirus; channel catfish virus; marine nodavirus including nervous necrosis virus and striped jack nervous necrosis virus; iridovirus including fish lymphocystis disease virus (ISAV) and white spot syndrome baculovirus (WSBV); penaeus monodonte baculovirus (MBV); white spot red vaculovirus and baculovirus penaei (BP); yellow headed virus; hepatopancreatic parvovirus (HPV); infectious hypodermal and haemato poietic necrosis virus (IHHNV).

In another embodiment, the bacterial pathogens include, but are not limited to, *aeromonas hydrophila*, *aeromonas salmonicida*, *yersinia ruckerii*, *pseudomonas aeruginosa*, *pseudomonas anguilliseptica*, *renibacterium salmoninarum*, *vibrio anguillarum*, *vibriosordalii*, *vibrio*, *pasteurellosis*, *edwardsiellosis ictaluri*, *edwardsiellosis tarda*, *sypophaga columnnari* and *rechettsia*. Also, another examples of the bacterial pathogens include causative bacteria of tuberculosis, gonorrhea, typhoid, meningitis, osteomyelitis, meningoccal septicemia, endometritis, conjunctivitis, peritonitis, pyelonephritis, pharyngitis, septic arthritis, cellulites, epiglottitis, salpingitis, otitis media, shigella dysentery and gastroenteritis.

In another embodiment, the parasitic pathogens include, but are not limited to *ichthyophthirius*, *Dactylogyrus*, *Gyrodactylus*, *myxobolus* sp, *ancylodiscoides*, *diplozoon* and the like.

The expression "increase in the expression level" means that the amount of a protein or transcription thereof increases when stressful environment is applied, as compared to the state when such stressful environment is not applied. The increased amount of the protein or transcription can be measured by conventional methods known to those skilled in the art. The methods for measuring an increased amount of a protein include, for example, SDS-PAGE and various immuno assays using a specific antibody, and an increased amount of transcription can be measured, for example, by northern blot.

The novel stress proteins according to the present invention and proteins immunologically related thereto exist in various organisms, not limited to fishes (Table 9). Therefore, the term "animal", as used herein, refers to living bodies in contrast to plants and can be construed as embracing fishes, birds, mammals, reptiles, rodents, primates and mankind. More specifically, the animals for the purpose of the present invention include



fin-fish such as flat-fish, shelfish, seam-bream, eel, trouts, carp, northwest salmon, atlantic salmon, rainbow trout, flounder and the like; moullusca such as oysters, mussels, clams, cockles, scallops, abalone and the like; echinoderm such as sea urchins, sea stars and the like; crustacea such as lobsters, shrimp, prawns, crabs, crayfish and the like; insects such as moth and the like; domestic cattles such as cow, lamb, pig, horse, dog, chicken, duck, turkey and the like; mammals such as mouse, rat, hamster and the like and other primates and mankind.

The term "chaperone activity" used herein means being able to aid folding of a substrate protein upon binding to a substrate protein so that the protein may reveal its native biological activities, to aid monomers to bind together with each other to form a polymer, to prevent a target protein from being aggregated into an inactive form, or to support the targeting process into the membrane.

The novel stress proteins according to the present invention and proteins immunologically related thereto are induced by various stresses such as heat shock, heavy metal and virus infection in numerous kinds of cells such as fishes, sellfish, human beings, mice, rats, hamsters, monkeys, larvae of moth, rice plants, crustaceans and cultured fish including flatfishes. At first, the inventors thought that the protein according to the invention was considered as one of known Hsp90 series since it is induced by stress. However, in the sequence analysis and study of its immunological properties, it was shown that the protein according to the present invention structurally and immunogenically differs from the known Hsp90 members.

In a particular embodiment according to the present invention, the protein represented by SEQ ID NO: 1 is cloned from CHES-214 cells. To compare the amino acid sequence of the protein NSP according to the present invention with that of other proteins, BLAST analysis was performed. The protein represented by SEQ ID NO: 1 according to

the present invention showed about 40% of sequence similarity with TraC protein, oligopeptide-binding protein (Fig. 3). Although the protein represented by SEQ ID NO: 1 has chaperone activity (FIG. 7), it does not show a high sequence similarity with known stress proteins. However, in a program search, the protein NSP according to the present invention was found to have ATP/GTP binding motif which is one of the basic properties of stress proteins, and to show a low sequence similarity with extracellular solute binding protein family 5, which has been reported to have chaperone activity in bacteria. Therefore, from the result of the sequence analysis it was confirmed that NSP, the stress proteins according to the present invention are novel stress proteins which have not yet been reported (FIG. 2).

The sequence similarity means that the ratio between residue of corresponding sequence and amino acid residue of the same candidate sequence, compared after aligning sequence and if required, introducing gap to obtain maximum ratio of identity to entire sequence. It was not construed that the homogeneity is lowered when N- or C-terminal is extended or inserted. The method for aligning sequence and computer program are known to those skilled in the art. The sequence homogeneity can be measured by using sequence analysis software such as Sequence Analysis Software Package, Genetics Computer Group, University of Wisconsin Biotechnology Center, 1710 University Ave., Madison, WI 53705.

The terms "protein" or "polypeptide" are intended to be used interchangeably. They refer to a chain of two (2) or more amino acids which are linked together with peptide or amide bonds, regardless of post-translational modification (e.g., glycosylation or phosphorylation).

Although the protein NSP of the present invention was first found in cell of trout infected with virus pathogen, considering the fact that its presence was observed in a very

wide range of animal and it is also expressed in a low level even in cells of other normal fishes such as salmon, rainbow trout, carp, which receive stress over a long period of time, the protein NSP of the present invention is believed to participate in the life preservation phenomenon (i.e., housekeeping) which life should maintain in normal environment.

5 Generally, stress proteins found in all animals are evolutionally preserved (Garry et al., Virology, 129, 319-332 (1983)).

As mentioned above, the protein NSP of the present invention comprises a protein denoted as SEQ ID NO: 1 and analogues, homologues, derivatives and fragments of polypeptide protein having at least one immunological epitope, which have substantially

10 homogeneous physiological activity.

As related point of view, the present invention provides a stress protein recognized by monoclonal antibody against the protein NSP and polypeptide fragments having at least one of immunological epitope.

“Homogeneous physiological activity” means that expression increases by external

15 stress such as pathogenic infection, atmospheric temperature increase, heavy metal influx etc, having chaperone activity, and the degree of expression in normal cell free from stress is lower than that in cell given with stress. Chaperone activity is defined as mentioned above.

In definition of the specification, “analogues” of protein denoted as SEQ ID NO: 1 refer to proteins which whole function and immunological property of the protein denoted as

20 SEQ ID NO: 1 according to the present invention are preserved, the one part of amino acid of which is substituted, deleted or added. Preferably, “substitution” of said amino acid is conservative substitution. Examples of conservative substitution of amino acid, which naturally exist, are as follows; aliphatic amino acid (Gly, Ala, Pro), hydrophobic amino acid (Ile, Leu, Val), aromatic amino acid (Phe, Tyr, Trp), acidic amino acid (Asp, Glu), basic

amino acid (His, Lys, Arg, Gln, Asn) and sulfur-containing amino acid (Cys, Met). Preferably, "deletion" of amino acid is positioned at the portion, which is not directly concerned with physiological activity of stress protein according to the present invention. For example, such analogues exist naturally, or may be manufactured by mutation of base  
5 sequence of gene or employing recombinant DNA technique, or may be directly obtained by synthetic method. Analogues according to the present invention shall possess at least one of antigen, which can induce antibody reacting with stress protein according to the present invention.

As other related point of view, analogues show at least about 80% of homogeneity  
10 with the amino acid sequence of protein denoted as SEQ ID NO: 1, and preserve the functional and structural features. Analogues of the protein NSP of the present invention more preferably show about more than 90%, most preferably at least about 90% of homogeneity with the amino acid sequence of protein denoted as SEQ ID NO: 1.

"Homologues" of protein denoted as SEQ ID NO: 1, a term used in the  
15 specification, are proteins derived from eukaryote lower and higher cell comprising shell fish besides fish cell, mankind, mice, rats, hamsters, monkeys, larvae of moth etc., which have the same sequence with that of the protein, or one of the amino acid sequence or more than that of amino acid residues are substituted with other amino acid residues while share the whole function and immunological property with the protein. For example, such  
20 homologues exist naturally, or may be manufactured by mutation of base sequence of gene or employing recombinant DNA technique, or may be obtained by synthetic method. Homologues of the protein shall possess at least one of antigen site, which can induce antibody reacting with the protein NSP.

"Derivatives" of protein denoted as SEQ ID NO: 1, a term used in the specification,

refer to polypeptides varied in more than one of physical, chemical, biological properties. Such variation comprises substitution, change, addition or deletion of amino acid; variation in pattern of lipidation, glycosylation or phosphorylation; reaction free amino, carboxyl or polypeptide with hydroxyl side group of amino acid residues, which other organic molecules and inorganic molecules are exist; and other variation resulted from change of primary, secondary or tertiary structure, but not limited thereto.

"Fragment" of protein denoted as SEQ ID NO: 1, a term used in the specification, refers to a part of polypeptide of protein, which comprises at least one immunological epitope. Immunological epitope is antigen determination group that induces immunoreaction. Preferred fragments of the present invention are those that, in animal including mankind, induce enough immunoreaction to prevent or alleviate serious infection with foreign pathogen.

The present invention provides the method for producing the stress protein. Especially preferred is utilizing genetic recombinant technique. DNA or RNA sequence encoding stress protein was expressed in suitable host cell to make cell lysate or mRNA of stress protein was in vitro translated, which can be purified by protein separation method known to those skilled in the art. Typically, to remove cell debris etc., after centrifuging the cell lysate or resultant product of in vitro translation, precipitation, dialysis, all sorts of column chromatography etc. are applied. Ion exchange chromatography, gel-permeation chromatography, HPLC, reverse phase-HPLC, SDS-PAGE, affinity column etc. are examples of column chromatography. For example, affinity column can be prepared by utilizing anti-NSP antibody.

Polypeptides of the stress protein according to the present invention, fragments and derivatives thereof can be prepared by employing one of the known organic chemical



method on peptide synthesis. Organic chemical method of peptide synthesis comprises coupling necessary amino acid by condensation reaction in homogeneous phase or under the aid of so-called solid phase. For most typical method on condensation reaction, there are carbodiimide method, azide method, mixed anhydride method and method using active ester, which are displayed in The Peptides Analysis, Synthesis, Biology Vol. 1-3, edited by Gross, E. and Meienhofer, J., 1979-1981, Academic Press Inc.

For especially suitable solid phase, there are p-alkoxybenzyl alcohol resin(4-hydroxy-methyl-phenoxy-methyl-copolystyrene-1% divinylbenzene resin) (Wang, J. Am. Chem. Soc., 95, 132 (1974)). After synthesis, peptides may be separated from said solid phase under gentle condition. After synthesis of purposed amino acid, separation of peptides from resin is executed by using trifluoroacetic acid containing scavenger such as triisopropyl silane, anisole or ethanedithiol, thioanisole. Reactive group not participating in condensation reaction is hydrolyzed or reduced by using acid or base, thereby being effectively protected by very easily removable group. Possible protective groups are detailed described in The Peptides, Analysis, Synthesis, Biology, Vol. 1-9, Gross, Udenfriend and Meienhofer edition, 1979-1987, Academic Press Inc.

Recombinant DNA technique is especially suitable for use in preparing substantially pure polypeptide according to the present invention. In the definition of the invention, "substantially pure" means that polypeptides or DNA sequence encoding polypeptides according to the invention substantially do not comprise other proteins derived from bacteria.

As other point of view, the present invention provides said protein NSP and DNA sequence encoding stress protein and polynucleotide sequence hybridized with them. Specifically, the invention provides polynucleotide sequences encoding NSP protein, which

are denoted as SEQ ID NO: 2, and polynucleotide sequence hybridized with them.

As related point of view, the invention provides nucleic acid molecules comprising a polynucleotide having at least 80%, 90% or 95% identity to the polynucleotide sequence encoding the protein NSP or otherwise consisting of such sequence. Specifically, the invention provides nucleic acid molecules comprising a polynucleotide having at least 80%, 90% or 95% identity to the polynucleotide sequence encoding the protein NSP, which is denoted as SEQ ID NO: 2, or otherwise consisting of such sequence.

As specific embodiment of the invention, gene encoding protein denoted as SEQ ID NO: 1 (hereafter, called as NSP gene) is separated and cloned. Said NSP gene comprises 2744 ea. of nucleotide and 2193bp size of ORF(open reading frame), which has characteristic of encoding amino acid 730 ea. from said genetic sequence. Normally, for separation and cloning of gene, traditional methods known to those skilled in the art may be employed. Particularly, in the present invention, NSP gene is separated from cDNA library of fish cell via screening process by preparing probe to NSP gene and using the prepared probe, and full length base sequence of said separated gene is identified by using RACE kit(GibcoBRL), followed by cloning said gene into expression vector, resulting in obtaining 186bp size of base sequence denoted as sequence ID number 9. Said 186bp size of DNA fragment is used in separation NSP gene from cDNA library of fish cell infected with virus.

Particularly, to make cDNA library, first of all, RNA is separated from fish cell, and cDNA is synthesized from mRNA. The synthesis of cDNA may use normal method known to those skilled in the art, and the synthesis employing ZAP-cDNA synthetic kit(Stratagene) is preferred. As exemplified in the invention, DNA should be tagged to use 186bp size of DNA fragment as a probe. For tagging method, tagging method employing radioisotope, enzyme etc. may be used, the tagging by using random primer kit(Stratagene)

is preferred.

In the examples of the invention, NSP cDNA was separated from cDNA library of fish cell by using said 186bp probe, which was cloned into cloning vector. For cloning vector, various kinds of vectors normally used are utilized, however, pBluescript vector(Stratagene) is used in the present invention. From the result of analyzing base sequence of NSP gene, only 1659bp size of sequence from 1089bp to 2744bp could be identified. In order to identify rest base sequence, normal methods known to those skilled in the art may be utilized, as in the present invention, RACE kit(GibcoBRL) may be utilized.

Polynucleotides encoding the stress protein of the invention and derivatives thereof denoted as SEQ ID NO: 1 may be synthesized by standard method known to those skilled in the art, for example, utilizing of automatic DNA synthesizer(e.g., available from biosearch, applied biosystems etc. For example, phosphorothioate oligonucleotide is synthesized by the method described in Stein et al., Nucl. Acids Res. 16:3209 (1989). Methylphosphonate oligonucleotide may be prepared by utilizing adjusted perforated glass polymer support (Sarin et al., Proc. Natl. Acad. Sci. U.S.A. 85:7448-7451 (1988)).

In a particular embodiment, the present invention provides a process for producing the protein NSP denoted as SEQ ID NO: 1 or immunologically related polypeptides thereof comprising steps of (a) inserting DNA sequence encoding said protein NSP or polypeptide into a vector having at least one of expression control sequences which is operatively linked to said DNA sequence and controls expression of said DNA sequence to form a recombinant expression vector, (b) transforming a host cell with the recombinant expression vector, (c) culturing the transformed cell in suitable media and conditions to express said DNA sequence, and (d) recovering substantially pure protein NSP from the culture.

As the related aspect, the particular embodiment of the present invention provides a

recombinant expression vector comprising at least one of polynucleotide sequences encoding polypeptide of the protein denoted as SEQ ID NO: 1 and transformed or transfected host cells with said expression vector.

As the method for separating cDNA encoding protein denoted as SEQ ID NO: 1(cDNA cloning), there is a method for isolating purposed cDNA by determining amino acid sequence of a part of the protein and hybridization method based on the base sequence corresponding to it, in addition, the method for isolating purposed cDNA by making cDNA library into expression vector and introducing it into cell, followed by screening purposed protein expressed (expression cloning method), although array of amino acid of protein is uncertain(D' Andrea et al., Cell, 57, 277-285 (1989); Fukunaga et al., Cell 61, 341-350 (1990)). In expression cloning method, cell, yeast, animal cell etc. have been used as host cell according to purpose.

The term "vector" means a DNA construct containing a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of the DNA in a suitable host. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may in some instances, integrate into the genome itself. In the present specification, "plasmid" and "vector" are sometimes used interchangeably. However, the invention is intended to include such other form of vector which serve equivalent function as and which are, or become, known in the art. Typical expression vectors for mammalian cell culture expression, for example, are based on pRK5(EP 307,247), pSV16B(WO 91/08291) and pVL1392(Pharmingen).

The phrase "expression control sequences" refers to DNA sequences necessary for the expression of an operably linked coding sequence in a particular host organism. Such

control sequences include promoter to execute transcription, optional operator sequence to control the transcription, sequence encoding suitable mRNA ribosome-binding site and sequence to control the termination of transcription and translation. For example, the suitable control sequences for prokaryotes include a promoter, optionally an operator  
5 sequence, and a ribosome binding site. Eukaryotic cells are known to utilize promoters, polyadenylation signals, and enhancers. In plasmid, factor exerting the greatest influence on the amount of gene expression is promoter. As promoter for high expression, SR promoter and promoter derived from cytomegalovirus etc. are preferably used.

In order to express DNA sequence of the invention, any of various expression  
0 control sequences may be used in vector. Examples of useful expression control sequences comprise, for example, SV40 or early and late promoters of adenovirus, lac system, trp system, TAC or TRC system, T3 and T7 promoters, major operator and promoter domain of phage lamda, regulatory domain of fd code protein, 3-phosphoglyceratekinase or promoter to other glycol dissolving enzyme, promoters of said phosphatase, for example, Pho5, promoter  
5 of yeast  $\alpha$ -hybridization system and other sequences and various combinations thereof of constitution and induction known to control gene expression of prokaryotes or eukaryotes or virus thereof. T7 RNA polymerase promoter  $\Phi 10$  may be usefully used to express the protein NSP in E. Coli.

Nucleic acid is "operably linked" when it is placed into a functional relationship  
0 with another nucleic acid sequence. This can be a gene and a regulatory sequence(s) which are connected in such a way as to permit gene expression when the appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequence(s). For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if is expressed as a preprotein that participates in the secretion of the polypeptide;



a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, “operably  
5 linked” means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adaptors or linkers are used in accordance with conventional practice.

10 The term “expression vector”, as used herein, generally indicates double-stranded DNA fragment as a recombinant carrier in which typically heterologous DNA fragment is inserted. The heterologous DNA means a hetero-type DNA which is not naturally occurring in host cells. Once the expression vector is incorporated into the host cell, it can be replicated regardless of host chromosomal DNA to produce several copies and heterologous DNA  
15 inserted into them.

As known to those skilled in the related art, to increase the expression level of gene transfected in host cell, corresponding gene should be operable connected to sequence for control transcription and decoding expression, which functions in selected expression host. Preferably, expression control sequence and corresponding gene are contained in one  
20 expression vector comprising virus selection marker and replication origin together. If expression host is eukaryotes, expression vector should further comprise expression marker useful in eukaryote expression host.

In order to express DNA sequence encoding the stress protein of the invention, various expression host/vector combinations may be used. Expression vectors suitable for

eukaryote host comprise expression control sequence derived from, for example, SV40, bovine papilloma virus, adenovirus, adeno-associated virus, cytomegalovirus and retrovirus. Expression vectors available for virus host comprise bacterial plasmid which materials obtained from *E. coli* are exemplified, such as pBluescript, pGEX2T, pUC vector, col E1, pCR1, pBR322, pMB9 and derivatives thereof, plasmid having wider range of host, such as RP4, phage DNA which can be exemplified as various phage lambda derivatives such as  $\lambda$ gt10 and  $\lambda$ gt11, NM989, and other DNA phage such as M13 and DNA phage filamentous single strain. Expression vector useful for yeast cell is 2  $\mu$  plasmid and derivatives thereof. Vector useful for insect cell is pVL 941.

In another aspect, the present invention provides a transformed or transfected host cell with the above expression vector. "Transformation" used herein means introducing DNA into an organism so that the DNA is replicable, either as an extrachromosomal element or by chromosomal intergration. "Transfection" used herein refers to the taking up of an expression vector by a host cell whether or not any coding sequences are in fact expressed.

The host cell of the invention may be prokaryotes or eukaryotes. Furthermore, hosts that introduction efficiency of DNA is high and expression efficiency of introduced DNA is high are generally used. Well known eukaryote and prokaryote hosts such as *E. coli*, *pseudomonas*, *bacillus*, *streptomyces*, fungi, yeast, insect cell such as *Spodoptera frugiperda*(SF9), animal cell such as CHO and mouse, Africa green monkey cell such as COS 1, COS 7, BSC 1, BSC 40 and BMT 10, and texture cultured human cell are examples of host cells which may be used. When cloning cDNA encoding stress protein of the invention, animal cells are preferred as host. The present invention exemplified CHSE-214, FHM, RTG-2 and EPC derived from fishes, but not limited thereto. When COS cell is used, SV40 large T antigen is expressed in COS cell, therefore, plasmid having replication

origin of SV40 are made to exist in episome of many copies in cell and higher degree of expression than normal case may be anticipated. Introduced DNA sequence may be obtained from the same species with host cell, or may be different species with host cell, or may be hybrid DNA sequence comprising certain heterogeneous or homogeneous DNA.

5           Of course, it should be understood that all vector and expression control sequence do not function equivalently in expression of DNA sequence of the present invention. Equally, all host function does not function equivalently to the same expression system. However, those skilled in the art can do suitable selection from various vector, expression control sequence and host without burden of excessive experiment and departing from the scope of the invention. For example, in selection vector, host should be considered because  
10           vector should be replicated in it. Replication number of vector, ability to control replication number and expression of other protein coded by corresponding vector, for example, antibiotic marker also should be considered. In selection expression control sequence, several factors should be considered. In relation to especially possible secondary structure,  
15           for example, relative strength, possibility to control and compatibility with DNA sequence of the invention etc. of sequence, should be considered. Single cell host should be selected by considering factors such selected vector, toxicity of product coded by DNA sequence of the invention, excretion property, ability to fold protein correctly, cultivation and fermentation requirements, easiness of purification product coded by DNA sequence of the invention from  
20           host etc. Within the range of these variables, those skilled in the art can select all sorts of vector/expression control sequence/host combination, which can express the DNA sequence of the invention in fermentation or large scale animal cultivation. As screening method when cloning cDNA of stress protein by expression cloning, binding method, panning method, film emulsion method etc. may be applied.

Furthermore, as particular embodiment, the present invention provides an antibody characterized by recognizing protein denoted as SEQ ID NO: 1 or analogues, homologues, derivatives or fragments of polypeptide protein having at least one of immunological epitope, and a preparing method thereof.

5           Still furthermore, as particular embodiment, the present invention provides hybridoma which produce an antibody characterized by recognizing protein denoted as SEQ ID NO: 1 or analogues, homologues, derivatives or fragments of polypeptide protein having at least one of immunological epitope.

10           Antigens of the invention are induced by immunotreatment of the protein NSP or immunologically related polypeptides (including analogues, homologues, derivatives thereof and fragments of polypeptide protein having at least of immunological epitope), or identified by reactivity with the protein NSP or immunologically related polypeptides.

15           Antibodies of the invention may be intact immunoglobulin molecule or fragments thereof containing intact antigen-binding site, comprising known fragments such as F(v), Fab, Fab' and F(ab')<sub>2</sub>. Antigens are produced by genetic engineering or chemical synthesis. Antibodies or fragments may be derived from animal, especially mammals, more specifically mice, rats, monkeys or mankind. Natural antibodies or fragments thereof, if necessary, recombinant antibodies or fragments of antibody are possible. Antibodies or fragments may be polyclonal antibody, preferably monoclonal antibody, and specific to  
20           many epitopes, preferably one epitope. Methods for preparing polyclonal antibody and monoclonal antibody are known to those skilled in the art. The specification quote a literature (Antibodies, A Laboratory Manual, supra, and D. E. Yelton, et al., Ann Rev. of Biochem., 50, 657-80 (1981)). Immunoreactivity of polypeptide of the invention can be determined by techniques well known to those skilled in the art, for example, immunoblot

analysis and ELISA.

Antibodies of the invention may form heterogeneous molecules from species (for example, fishes and mankind) and other immunoglobuline sequence, or may be constructed with parts of light and heavy chain sequence of immunoglobulin from same species. Such antibodies become molecule having multibinding specificity, for example, bifunctional antibodies prepared by techniques known to those skilled in the art, i.e. techniques selected from production of hybridoma, disulfide exchange, chemical cross-linking, addition of peptide binder between two of monoclonal antibodies and so on. Furthermore, antibodies of the invention may be, for example, human monoclonal antibody produced by immortal humane cell, SCID-hu mouse or animal able to produce human antibody or replicated human immunoglobulin.

In short, those skilled in the art can change biological property or other properties of antibody of the invention from the technique of the invention, by methods of stability or half-life, immunological property, toxicity, increasing or reducing of hydrophilicity or molecular weight of given antibody molecule.

The monoclonal antibodies of the invention may be obtained by following method, as particular embodiment. In other words, for immunogenic antigen necessary to make monoclonal antibody, natural protein NSP or genetic recombinant protein NSP may be used. Hybridoma is prepared by immunizing mammals with each antigen, or by normal method comprising fusing lymphocyte immunized by in vitro method with myeloma cell line and so on. Each antigen highly purified is used to this hybridoma solution, and by solid phase ELISA is selected antibody-producing hybridoma that recognize each antigen. The obtained hybridoma is cloned, and the obtained stable antibody-producing hybridoma is respectively cultivated to purposed antibody. Mice and rats are used in preparing hybridoma.



A general method for immunity comprises the steps: antigen is diluted in suitable solvent, for example, physiological saline solution etc. with suitable concentration, and then the solution is administered intravenous or intra abdominal, and if necessary, Freund adjuvant is administered together, administered to animal about 3 to 4 times at an interval of 1 to 2 weeks. Resultant immune animal is dissected 3-4 days after the final immunization, and spleen cell obtained from spleen removed is used as immune cell. For myeloma derived mice cell fused with immune cell, for example, p3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, F0, P3x63 Ag8. 653 and S194 etc. may be used. Furthermore, for cells derived from rats, cell line such as R-210 etc. may be used. Human type antibody may be prepared by in vitro immunization of human B lymphocyte, and cell fusion with human myeloma cell and cell line transformed by EB virus.

For fusion immune cell with myeloma cell line, known method, for example, methods of Koehler and Milstein are generally used, but electric pulse method using electric pulse also is available. Immunolymphocyte and myeloma cell line are mixed in the ratio of cell number normally employed, and then fusion treatment is achieved by adding polyethyleneglycol to FCS unmerged medium for cell culture normally used, and then fusion cell(hybridoma) is screened by culturing with FCS-added HAT selection medium. Thereafter, hybridoma-producing antibody is selected by detection method typically employed such as ELISA, plague method, ouchterlony method, agglomeration method etc., and then stable hybridoma is collected. Such collected hybridoma can do subculture by normal culturing method, and if necessary, may be cryopreserved. Hybridoma is cultured by normal method to recover culture solution or the culture solution is transplanted into abdomen of mammals to recover antibody from abdominal dropsy. Culture solution or antibody in abdominal dropsy may be purified by salting out, ion exchange and gel filtration

chromatography, protein A or protein G affinity chromatography etc., which are normally employed. Almost all of monoclonal antibodies obtained by such method can specifically recognize natural protein NSP as well as analogues, derivatives and/or homologues thereof. With radioisotope or enzyme tag, such antibodies can be measured by measuring method known as radio immunoassay (RIA) and enzyme immunoassay (EIA).

As specific embodiment of the invention, polyclonal antibody that recognizes protein denoted as SEQ ID NO: 1 can be obtained as follows. Host cell that expresses the protein NSP is pulverized, and the resultant product is purified by OCIF fixation column and gel filtration chromatography, to obtain natural protein NSP, which shall be used as antigen for immunization. Furthermore, NSP cDNA(SEQ ID NO: 2) is inserted into expression vector by normal method, and the formed recombinant plasmid is expressed to animal cell such as CHO cell, BHK cell, Namalwa, COS-7 cell etc., insect cell or E. coli etc., followed by purification with the same method, therefore, genetic recombinant protein NSP may be obtained, which is properly used as antigen for immunization.

Alternatively, to 5' terminal upstream of NSP cDNA(SEQ ID NO: 2) is added base sequence encoding known signal arrangement derived from other excreted protein, which is inserted into expression vector by the same genetic engineering method to be expressed by having all kinds of animal cell, insect cell or E. coli etc. as host, and then the resultant protein is purified, to obtain antigen for immunization. The antigen for immunization obtained as above is dissolved in phosphate buffered physiological saline solution(PBS), and if necessary, equivalent volume of Freund adjuvant is mixed to be emulsified, which is administered subcutaneous to animal at interval of about 1 week, to immunize several times. Threshold value of antibody is measured, and then at the time of maximum value of antibody, additional inoculation is executed, and 10 days after administration, blood-

gathering is executed to all. Ammonium sulfate fraction precipitation is achieved to  
obtained antiserum, and then globulin fraction is purified by anion exchange  
chromatography, or the antiserum is 2-fold diluted with binding buffer(Biorad), and then the  
diluted antiserum is purified by protein A or protein G sepharose column chromatography,  
5 resulting in obtaining anti- NSP polyclonal antibody.

Antibodies obtained as above that neutralize biological activity of stress protein are  
utilized as a medicine, i.e. pharmaceutical composition for prevention and/or treatment of  
infectious disease by pathogen, or antibody for immunological diagnosis of such infectious  
disease. Antibodies of the invention may be orally or parenterally administered after being  
10 formulated. Formulation comprising the antibody of the invention is safely administered to  
man or animal as a pharmaceutical composition containing antibody that recognize stress  
protein of the invention as a active ingredient.

As specific embodiment, the invention provides pharmaceutical composition for  
prevention and/treatment diseases specifically shown by the expression of the protein NSP,  
15 characterized by containing antibodies which recognize the protein NSP, in therapeutic or  
preventive effective amount, and pharmaceutically acceptable carriers. "Diseases  
specifically shown by the expression of the protein NSP", the term used in the specification,  
indicates all infectious diseases occurred by infection of mammal with viroous, bacterial or  
parasitic pathogen.

20 The carrier used in the pharmaceutical composition of the invention comprises  
pharmaceutically acceptable carrier, adjuvant and vehicle, collectively called  
"pharmaceutically acceptable carrier". Pharmaceutically acceptable carrier that can be used  
in the pharmaceutical composition of the invention comprises ion exchange, alumina,  
aluminium stearate, lecithin, serum protein (e.g., human serum albumin), buffering material

(e.g., various phosphate, glycine, sorbic acid, potassium sorbate, partial glyceride mixture of saturated plant fatty acid), water, salt or electrolyte (e.g., protamine sulfate, sodiumdihydrogen phosphate, potassium hydrogen phosphate, sodium chloride and zinc salt), colloidal silica, magnesium trisilicate, polyvinyl pyrrolidone, cellulose-containing substrate, polyethylene glycol, sodium carboxymethylcellulose, polyarylate, wax, polyethylene-polyoxypropylene-blocked polymer, polyethylene glycol and wool fat etc., but not limited thereto.

The administration route of the pharmaceutical composition according to the invention comprises oral, intravenous, intramuscular, intraarterial, intra bone marrow, intrathecal, intracardiac, dermal, subcutaneous, intraabdominal, intranasal, intestinal tract, local, hypoglossal or rectal injection, but not limited thereto.

Oral and parenteral administration are preferred. The term "Parenteral", as used herein, comprises subcutaneous, intradermal, intravenous, intramuscular, intrajoint, intrabursa, intrasternal, intrathecal, intrafocus and intracranial injection technique.

As preferred embodiment, pharmaceutical composition for oral administration may be formulated by admixing active ingredient with solid phase excipient, and formulated as granule form to prepare tablet or sugarcoated tablet. For suitable excipient, sugar form such as lactose, sucrose, mannitol and sorbitol or starch from corn, flour, rice, potato or other plants, cellulose such as methyl cellulose, hydroxypropylmethyl-cellulose or sodium carboxymethylcellulose, carbohydrates such as gums comprising arabic gum, tagakans gum or protein filler such as gelatin, collagen may be used. If necessary, disintegrant or solvent in form of salt respectively, such as cross-linked polyvinylpyrrolidone, agar and alginic acid or sodium alginate may be added.

As preferred embodiment, for parenteral administration, pharmaceutical

composition of the invention may be formulated to water-soluble solution. Preferably, physically suitable buffered solution such as Hank's solution, Ringer's solution or physically buffered brine may be used. Substrates that can increase the viscosity of suspension sodium carboxymethyl cellulose, sorbitol or dextran may be added to water-soluble injection suspension. In addition, suspension of active ingredient may be formulated to suitable oily injection suspensions. Suitable lipophilic solvent or carrier comprises fatty acid such as sesame oil or synthetic fatty acid ester such as ethyl oleate, triglyceride or liposome. Also, polycationic amino polymers may be used as carrier. Optionally, suspension may use suitable stabilizer or agent to increase the solubility of compound and prepare solution in high concentration.

The pharmaceutical composition may be a form of formulation for sterilization injection as aqueous or oily suspension for sterilization injection. Such suspension may be formulated by using suitable dispersant or wettable agent(e.g., Tween 80) and suspension agent according to the method known to those skilled in the art. Formulation for sterilization injection may also be nontoxic parenterally acceptable diluent or sterilization injection solution in solvent or suspension(e.g., solution in 1,3-butanediol). For acceptably used vehicle and solvent, mannitol, water, Ringer's solution and isotonic sodium chloride solution are comprised. Furthermore, sterilized nonvolatile oil is normally used as solvent or suspension medium. For such purpose, any nonvolatile oil having low degree of property on stimulation, comprising synthetic mono or diglyceride, may be used. Fatty acid such as oleic acid and glyceride derivatives thereof is also useful for injection formulation likewise pharmaceutically acceptable natural oil (e.g., olive oil or castor oil), especially polyoxyethylated thereof.

The pharmaceutical composition of the present invention may be also administered



in form of suppository, for administration to rectal. Such composition may be prepared by mixing the compound of the invention with suitable nontoxic excipient that is solid at room temperature but liquid at the temperature of rectal. Such substances comprise cocoa butter, beeswax and polyethylene glycol, but not limited thereto.

5           When the pharmaceutical composition according to the invention is locally applied to skin, the pharmaceutical composition should be formulated to suitable ointment that contains an active ingredient suspended or dissolved in carrier. The carrier, for topical application of the composition of the invention, comprises mineral oil, fluid paraffin, white Vaseline, propylene glycol, polyoxyethylene, polyoxypropylene compound, emulsified wax  
10 and water, but not limited thereto. Alternatively, pharmaceutical composition may be formulated to suitable lotion or cream ointment that contains active compound suspended or dissolved in carrier. Suitable carrier comprises mineral oil, sorbitan monostearate, polysolvate 60, cetyl ester wax, cetearyl alcohol, 2-octyldodecanol, benzyl alcohol and water, but not limited thereto. Pharmaceutical composition of the invention may be also locally  
15 applied by rectal suppository or as suitable enema to lower body intestinal tract, dermal patch locally applied also is comprised in the present invention.

The pharmaceutical composition may be administered by intra nasal aerosol or inhalation. Such composition is prepared according to well known technique in pharmaceutical field, by using of benzyl alcohol or other suitable preservative, absorption  
20 promoter to enhance bio availability, fluorocarbon and/or solubilizer or dispersant well known in the art to be prepared as solution in brine.

The compound of the invention may be used by being mixed normal anti-inflammatory or matrix metaloprotease inhibitor, lipoxigenase inhibitor and inhibitor of cytokin except IL-1  $\beta$ . Compound of the invention also may be administered to prevent or

suppress IL-1 mediated disease symptom such as inflammation in combination with immunocontrol agent(e.g., bropyrimine, anti-human  $\alpha$  interferon antibody, IL-2, GM-CSF, methionine enkephalin, interferon  $\alpha$ , diethyldithiocarbamate, humor death factor, Naltrexone and rEPO) or prostagladine. When the compounds of the invention are administered in combination with other therapeutic formulation, those may be administered to patient consecutively or simultaneously. Otherwise, the pharmaceutical composition according to the invention may be achieved by mixing with the compound of general formula (1) and other therapeutic or preventive agent as formerly mentioned.

Pharmaceutical compositions of the present invention may be used in treating infectious diseases, especially diseases related with virus infection. Diseases treated or prevented by pharmaceutical composition of the invention comprise tuberculosis, gonorrhea, typhoid, meningitis, osteomyelitis, meningococcal infection, endometritis, conjunctivitis, peritonitis, pyelitis, pharyngitis, septicemic arthritis, cellulitis, laryngitis, salpingitis, otitis media, cold, influenza, enteritis, dysentery and gastritis, but not limited thereto.

The term "therapeutically effective amount" indicates, in case of man, to use for treating said symptoms, about 1mg to about 10mg per body weight kg per day of dose level (typically about 50mg to about 500mg/patient/day). In case of fishes, therapeutically effective amount of composition used for treating the infection by pathogen is 2-10 mg/kg. In case of livestock, therapeutically effective amount of composition used for treating the infection by pathogen is 4-10mg/kg.

The term "preventively effective amount" indicates, in case of man, to use for preventing said symptoms, about 0.1mg to about 1mg per body weight kg per day of dose level (typically about 5mg to about 50mg/patient/day). In case of fishes, preventively effective amount of composition used for treating the infection by pathogen is 0.2-1mg/kg. In

case of livestock, preventively effective amount of composition used for treating the infection by pathogen is 0.4-1mg/kg.

The amount of antibody that can be combined with a carrier to a unit dosage form may vary according to host treated and specific administration mode. For example, formulation for administering orally to man may contains 50mg to 500mg of antibody in combination with pharmaceutically accepted carrier in suitable and convenient amount that can account for about 20 to about 40% of total composition. Single volume type generally contains about 10mg to about 50mg of antibody.

However, it will be understood that particular effective amount to particular patient can be changed according to various factors comprising activity of particular compound used, age, body weight, general health, sex, regular food, administering time, administering route, excretion rate, medicine combination and serious illness of particular diseases to be prevented or treated. Pharmaceutical composition according to the invention may be formulated into pill, sugarcoated tablet, capsule, fluid, gel, syrup, slurry, and suspension.

The pharmaceutical composition according to the invention may be administered to branchial pouch or digestive organ if administered to subcutaneous cell of fishes. Injection may be carried out intramuscular cell in muscular texture or other cell, also internal cell in abdominal cavity.

The monoclonal antibody in pharmaceutical composition adsorbs glass vessel such as vial etc. and syringe tub etc. and also is unstable, and its activity is easily reduced by various physical chemical factors, e.g., heat, pH and humidity and so on. Therefore, to be formulated into stable form, stabilizer, pH controller, buffer, solubilizer, surfactant etc. are added. For stabilizer, amino acids such as glycine, alanine etc., saccharide such as dextran 40 and mannose etc., saccaride alcohol such as sorbitol, mannitol, xylitol etc. are mentioned,

of which also can be used in combination of more than 2 kinds. Amount of stabilizer added is preferably 0.01 to 100 fold, particularly 0.1 to 10 fold to the weight of antibody. As a result of addition these stabilizer, preservative stability of liquid phase formulation or lyophilized formulation may be enhanced. For buffer, phosphate buffer, citrate buffer etc. are listed. Buffer adjusts pH of aqueous solution after redissolution of liquid phase formulation or lyophilized formulation, and contributes to stability and solubility of antibody. Amount of buffer added is preferably for example, 1 to 10mM, to the quantity of after redissolution of liquid phase formulation or lyophilized formulation. For surfactant, polysolvate 20, pulluronic F-68, polyethyleneglycol may be used, especially preferably, polysolvate 80 is mentioned, of which also can be used in combination of more than 2 kinds.

As mentioned above, high molecular protein such as antibody is easily adsorbed to the material of container, glass and resin etc., therefore, by the addition of surfactant, adsorption of antibody to the container after redissolution of liquid phase formulation or lyophilized formulation may be prevented. Amount of surfactant added is preferably 0.001 to 1.0%, to the weight of water after redissolution of liquid phase formulation or lyophilized formulation. As mentioned above, in preparing formulation of antibody of the invention, stabilizer, buffer or anti-adsorption agent may be added thereto, but, especially in case of being employed as medical or veterinary injection, osmotic pressure ratio as acceptable osmotic pressure ratio is preferably 1 to 2. Osmotic pressure ratio is controlled according to the increase and reduction of sodium chloride in formulation. Antibody content in the formulation may be properly adjusted according to applied diseases, administered route etc., amount administered to man of human type antibody depend on the affinity of antibody to human protein NSP, i.e., depend on the dissociation constant ( $K_d$  value) to human protein NSP, if affinity is high ( $K_d$  value is low), with less dose as much, the medicine can take

effect.

As related point of view, a specific embodiment of the invention provides pharmaceutical composition to treat diseases specifically shown by the expression of the protein NSP, comprising the protein NSP or immunologically related fragments thereof or DNA molecules encoding them. Preparing method and administering method etc. of the pharmaceutical composition is as abovementioned. The amount administered to patient may be determined by employing of known technique by those skilled in the art, according to activity of protein or nucleic acid molecules used, age, weight, health condition, sex, administration route, combination of medicine and kind of specific disease and so on.

Furthermore, as related point of view, specific embodiment of the invention provides pharmaceutical composition to treat diseases specifically shown by the expression of the protein NSP, comprising promoter or inhibitor of activity or expression of the protein NSP. The inhibitor suppressing expression of the protein NSP comprises antisense oligonucleotide, ascorbic acid and NOS inhibitor, but not limited thereto. Preparing method and administering method etc. of the pharmaceutical composition is as abovementioned. The amount administered to patient may be determined by employing of known technique by those skilled in the art, according to activity of protein or nucleic acid molecules used, age, weight, health condition, sex, administration route, combination of medicine and kind of specific disease and so on.

In the present invention, it has been found that expression of the protein NSP increase in all sorts of cancer texture by employing antibody against NSP protein or polynucleotide encoding NSP protein. As such point of view, the present invention provides composition to diagnose diseases followed by increase of the protein NSP expression, comprising antibody against NSP protein or polynucleotide encoding NSP



protein. The term used here, "diseases followed by increase of the protein NSP expression" indicates all infectious diseases as well as all sorts of cancer, which occur by infection of mammal with viroous, bacterial or parasitic pathogen as described above. Representative cancer comprises breast cancer, large intestine cancer, stomach cancer, ovarian cancer, lung cancer, kidney cancer, prostate cancer and brain tumor, bur not limited thereto.

The inventors have found that NSP protein generally express more in cell infected with pathogen than in normal cell. In relation to this, it is believed that significantly higher level of the protein NSP gene expression can be detected in body fluids(e.g., serum, blood plasma, urine, synovia or spinal fluid or specific cell or texture(e.g., bone marrow)) collected from patient having infectious diseases by such pathogen, compared with "standard" NSP gene expression level(i.e., NSP expression level in body fluids or texture of individual not having such diseases). Therefore, the present invention provides diagnosing method, which comprises (a) assaying NSP gene expression level in cell, texture or body fluids of animal, (b) monitoring diseases by comprising comparing NSP gene expression level with standard NSP gene expression level. At this time, increase of assayed NSP gene expression level compared with standard expression level means diagnosing of diseases.

An additional specific embodiment of the invention provides a method for detecting pathogenic infection from biological sample of animal by employing antibody against NSP protein or polynucleotide encoding the protein and diagnosing kit. The method comprises (a) contacting the biological samples with antibody against the protein NSP, (b) detecting pathogenic infection from the biological samples by probing the presence of corporation fused antibody against the protein NSP and the protein NSP in the samples, from the samples. As other embodiment, the method of the invention comprises (a) reacting biological samples with polynucleotide encoding the protein NSP, (b) detecting NSP

expression gene or fragments thereof from the biological samples, which react with polynucleotide encoding the protein NSP. Suitable biological sample comprises serum, blood plasma, urine, synovia or spinal fluid or specific cell or texture(e.g., bone marrow). As one embodiment, diagnosing kit comprises antibody against NSP polypeptide of the invention or polynucleotide encoding the protein NSP or mixture thereof in combination with detection reagent.

Furthermore, a specific embodiment provides a method for detecting pathogenic infection, comprising (a) collecting a biological sample from a patient, (b) contacting the sample with more than two oligonucleotide primer(at least one of oligonucleotide primers is specific to polynucleotide sequence encoding NSP protein, especially a protein denoted as SEQ ID NO: 1) by polymerase chain reaction, and (c) detecting polynucleotide sequence amplified under the presence of oligonucleotide primer from the samples.

As other point of view, a specific embodiment of the invention provides a method for detecting pathogenic infection from patient, comprising (a) collecting biological samples from patient, (b) contacting the samples with oligonucleotide probe specific to polynucleotide sequence encoding NSP protein of the invention, (c) detecting polynucleotide sequence hybridized with oligonucleotide probe from the samples. As one embodiment, oligonucleotide probe comprises polynucleotide sequence encoding the protein NSP or about more than 15 of continuous nucleotide from the sequence hybridized therewith.

The probe or primer specific to polynucleotide sequence encoding NSP protein of the invention, especially protein denoted as SEQ ID NO: 1 has at least 16, preferably at least 24 of nucleotides. Using standard hybridization method may use probe or primer of the invention in screening for cDNA library.

cDNA encoding protein denoted as SEQ ID NO: 1 may be used to design PCR

primer or amplify nucleic acid sequence. Those skilled in the art, and in diagnostics normally execute PCR method, the use have been known and allowed. Performing procedure of PCR method is displayed in 'PCR Protocols: A Guide to Methods and Applications', Innis, M.A., et al. Eds. Academic Press, Inc. San Diego, California (1990), which is incorporated herein as reference. The application of PCR method is displayed in 'Polymerase Chain Reaction', Erlich, H.A., et al., Eds. Cold Spring Harbor Press, Cold Spring Harbor, New York (1989), which is incorporated herein as reference. With a part of simple rule, effective primers are designed. Normal primer is nucleotides having 50% to 60% G+C composition of 18 to 28 each. Whole primer is preferably complementary with sequence to be hybridized. Preferably, primer produces PCR product of 100 bp to 2000 bp. However, more than 50 bp to 10 kb of PCR product may be produced.

The PCR method provides 5' and 3' primer to hybridize the sequence present in nucleic acid molecules, thereby making it possible to rapidly produce multi copy number of nucleotide sequence, and to produce complementary DNA strain, isolated nucleotide and enzyme charged into complementary base to nucleotide sequence are additionally provided between primer having isolated nucleotides. The enzyme is inserted into complementary sequence adjacent to primer. When all of 5' primer and 3' primer are hybridized against nucleotide sequence on complementary strain of the same fragment of nucleic acid, exponential amplification of specific double strand product occurs. When only single primer is hybridized into nucleic acid molecule, linear amplification produce single strand product of variable length.

The present invention provides a promoter that enhances the function of NSP protein and a inhibitor that suppresses or reduces the function.

The inhibitor comprises substances that suppress or obstruct the activity of NSP

protein, and also substances that suppress or obstruct the expression. As one embodiment, the inhibitor comprises small organic molecules, peptides, polypeptides and antibodies, which bind the stress protein of the invention, thereby suppress or eliminate activity thereof. Furthermore, the inhibitor may be small organic molecules, peptides, polypeptides or antibody inhibiting the binding of stress protein with intracellular protein, thereby suppressing the function of stress protein.

The inhibitor suppressing of the protein NSP according to the invention comprises substances that suppress the transcription or translation of NSP gene. The expression of the invention is preferably ascorbic acid and NOS (Nitric Oxide Synthase) expression inhibitor. NOS expression inhibitors comprise NAME, LMMA(N(G)-monomethyl-L-arginine), and aminoguanidine. An especially preferred inhibitor is NAME.

The present invention provides antisense molecules of the protein NSP. Especially, antisense molecules may be usefully used as an A protein NSP with chaperone activity which is expressed in an increased level in animal cells stimulated by external stresses inhibitor to suppress the expression of the NSP protein of the invention. Antisense technique may be used in controlling the gene expression through antisense DNA or RNA or the formation of triple stranded helix. Antisense technique is displayed, for example, in literature Okano, J. Neurochem. 56:560 (1991); "Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression." CRC Press, Boca Raton, FL (1988). The formation of triple stranded helix is displayed, for example, in literature Lee et al., Nucleic Acids Research 6:3073 (1979); Cooney et al., Science 241:456 (1988); and Dervan et al., Science 251: 1360 (1991). These methods are based on the binding of complementary DNA or RNA with polynucleotide. For example, 5' code part of polynucleotide encoding polypeptide domain according to the present invention may be used to design antisense RNA oligonucleotide the

length of which is about 10 to 40 base pairs. DNA oligonucleotide is designed to be complementary to the domain of gene involved in transcription, thereby suppressing the transcription and production of NSP protein. Antisense RNA oligonucleotide is in vivo hybridized with mRNA to block the decoding of mRNA molecule to NSP polypeptide.

5 Oligonucleotide as abovementioned is also transmitted to cell, followed by in vivo expressing antisense RNA or DNA, resulting in inhibition of production of stress protein.

In one embodiment, antisense nucleic acid denoted as SEQ ID NO: 1 of the invention is intracellularly produced by transcription from foreign sequence. For example, the vector or part thereof is transcribed to produce antisense nucleic acid(RNA) of the invention. Such vector contains sequence encoding NSP antisense nucleic acid. Such

10 vector is preserved as episome or integrated into chromosome as far as it is transcribed to produce purposed antisense RNA. Such vector can be made by standard recombinant DNA technique in the art. Vectors are to be used for replication and expression in vertebrate cell, which may be plasmid, virus etc. known in the art. The expression of

15 sequence encoding the protein NSP or fragments thereof is possible in any promoter known to the field as functioned in vertebrate, preferably human cell. Such promoter may be inductive or constructive. These promoters comprises comprise SV40 early promoter domain (Bernoist and Chambon, Nature 29:304-310 (1981)), promoter contained in 3' long term repetition of Rous sarcoma virus (Yamamoto et al., Cell 22:787-797 (1980)), herpes

20 thymidine promoter (Wagner et al., Proc. Natl. Acad. Sci. U.S.A. 78:1441-1445 (1981)), control sequence of metallothionein gene (Brinster, et al., Nature 296:39-42 (1982)) etc., but not limited thereto.

The antisense nucleic acid of the invention comprises complementary sequence to at least a part of RNA transcription. However, absolute complementarity is preferred, but



not always required. Sequence "complementary to at least a part of RNA", mentioned in the specification means sequence having enough complementarity to hybridize its RNA to be able to form stable double strand. Therefore, as one embodiment, in case of double strand NSP antisense nucleic acid, single strand of double strand DNA may be tested or  
5 assayed on the forming of triple strand. Ability to hybridize depends on both the degree of complementarity and the length of antisense nucleic acid. Generally, the more hybridized nucleic acid is big, the more base unconformity with NSP RNA that nucleic acid contain and can be stable double strand(or triple strand) exist. Those skilled in the art may grasp the acceptable degree of unconformity by employing standard procedure that determines the  
10 melting point of hybridized complex.

Oligonucleotide complementary to 5' terminal (e.g., 5' undecoded sequence so far, comprising AUG start condon) of message should most effectively function when suppressing decoding. However, sequence complementary to 3' undecoded sequence of mRNA is presented to be also effective in suppressing decoding of mRNA. Therefore, to  
15 oligonucleotide complementary 5'- or 3'- undecoded and uncoded domain of NSP may be used for antisense method to suppress decoding of intrinsic NSP mRNA. Oligonucleotide complementary to 5' undecoded domain of mRNA should comprise complement of AUG start codon. Antisense oligonucleotide complementary to mRNA code domain is less effective decoding an inhibitor, but may be used according to the present invention.  
20 Antisense nucleic acid should be nucleotide of more than 6 ea in length, regardless of whether is designed to be hybridized to 5'-, 3'- or code domain of NSP mRNA or not, preferably, oligonucleotide consisting of nucleotide of 6 to about 50 ea in length. From specific point of view, oligonucleotide consists of more than 10 ea of nucleotide, more than 17 ea of nucleotide, more than 25 ea of nucleotide or more than 50 ea of nucleotide.

The antisense oligonucleotide includes, but is not limited to, at least one modified base residue selected from a group consisting of 5-fluorourasil, 5-bromourasil, 5-chlorourasil, 5-iodourasil, hypoxanthine, xanthine, 4-acethylcytosine, 5-(carboxyhydroxymethyl)urasil, 5-carboxymethylaminomethyl-2-thiouridine, 5-carboxymethylaminomethylurasil, dihydrourasil, beta-D-galactosylquinosine, inosine, N-6-isopentenyladenine, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanin, 2-methyladenin, 2-methylguanin, 3-methylcytosine, 5-methylcytosine, N6-adenin, 7-methylguanin, 5-methylaminomethyluracil, 5-methoxyaminomethyl-2-thiouracil,  $\beta$ -D-maanocilquiosin, 5-methoxycarboxymethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyl adenin, uracil-5-oxyacetic acid(v), butoxin, pseudouracil, quiosin, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid(v), 5-methyl-2-thiouracil, 3-(3-amino-3-N-2-carboxypropyl)uracil, (acp3)w and 2,6-di aminofurine.

The antisense oligonucleotide includes, but is not limited thereto, at least one sugar residue selected from a group consisting of arabinose, 2-fluoroarabinose, xylulose and hexose.

In another embodiment, the antisense oligonucleotide include, but is not limited thereto, at least one modified phosphate back bone selected from a group consisting of phosphorothioate, phosphorodithioate, phosphoroamidothioate, phosphoroamidate, phosphorodiamidate, methylphosphonate, alkylphosphotriester and formacetal or analogues thereof.

Further, in another embodiment, antisense oligonucleotide is  $\alpha$ -anomer oligonucleotide.  $\alpha$ -anomer oligonucleotide forms complementary RNA and specific double strandedness hybrid, and in contrast with normal  $\beta$ -unit, strandedness run in parallel each

other (Gautier et al., Nucl. Acids Res. 15:6625-6641 (1987)). Oligonucleotides are 2'-O-methylribonucleotide (Inoue et al., Nucl. Acids Res. 15:6131-6148 (1987)) or chimera RNA-DNA analogues (Inoue et al., FEBS Lett. 215:327-330 (1997)).

Although antisense nucleotides complementary to code domain sequence of the protein denoted as SEQ ID NO: 1 may be used, antisense nucleotides complementary to transcribed undecoded domain are most preferred.

The potential inhibitor according to the invention comprises catalytic RNA or ribozyme (reference: disclosed in 4 Oct 1990, PCT international disclosure WO90/11364; Sarver et al., Science 247:1222-1225 (1990)). Using of ribozyme that cut mRNA at site-specific recognition sequence may disintegrate NSP mRNA, but hammer-head ribozyme is preferably used. Hammer-head ribozyme adjoins domain forming base pair complementary, to target mRNA to cut mRNA at directed position. Only requirement is that target mRNA have two base 5'-UG-3' sequence. Preparing and production of hammer-head ribozyme are well known in the art, which is more detailed described in literature Haseloff and Gerlach, Nature 334:585-591 (1988). A lot of potential hammer-head ribozyme cutting sites are present in nucleotide sequence of NSP. Preferably, the ribozyme is engineeringly operated so that cutting recognition site should be positioned near 5' terminal of NSP mRNA, that is, efficiency increases and intracellular accumulation of inactive mRNA transcription is minimized.

Like the antisense method, ribozyme of the invention may consist of modified oligonucleotide(e.g., for enhanced stability, target aiming etc.), and should be in vivo transmitted to cell expressing the protein NSP. DNA product encoding ribozyme may be inserted in cell by the same method as formerly described on the insertion of DNA encoding antisense. Preferred method for delivering comprises employing DNA product "encoding"

ribozyme under control of strong constructive promoter (e.g., pol III or pol II promoter), so that transfected cell destroys intrinsic NSP message and provides enough amount of ribozyme to suppress decoding. Unlike antisense molecule, ribozyme is catalyst; therefore, lower intracellular concentration is required for efficiency.

5           The invention provides a method for screening compounds suppressing the expression of gene encoding said NSP protein, comprising contact with any compound, cells that contain genes encoding NSP protein and express them, screening compounds reducing the expression of said gene encoding NSP protein. Methods for measuring excessive gene expression are known. "Expression of gene" mentioned above means that gene encoding  
10   NSP protein is transcribed to mRNA or protein is produced from the transcription. Therefore, excessive gene expression may be measured by measuring the amount of NSP protein, or the amount of transcription of NSP gene. For method of measuring the amount of NSP protein, immunoassay utilizing antibody specific to NSP is exemplified. For immunoassay, RIA, ELISA, dot blot, western blot, sandwich assay etc. may be used. For  
15   method of measuring the amount of transcription of NSP gene, northern blot may be recommended. To measure the expression of NSP gene of the invention, the reporter gene system may be employed. For representative reporter gene, gene encoding fluorescent protein, luciferase and  $\beta$  glucuronidase is exemplified.

          Furthermore, the present invention provides a method for screening a substance  
20   suppressing the activity of said stress protein, comprising contacting NSP protein with any compound, screening compounds suppressing chaperone activity of said stress protein. Methods for measuring the activity of stress protein are known. For example, method for super high screening the inhibitor of the invention is well known, by execution of High Throughput Screening (HTS) (Devlin, J.P. Eds. High Throughput Screening; Marcel

Dekker, New Your, 1997) to compound already existed or extracted from natural substances, or derivatives thereof, to effectively execute screening of compounds inhibiting or suppressing the chaperone activity of said stress protein. Those skilled in the art also may employed known combinatorial chemistry technology to obtain large quantities of inhibitor of protein of the invention in short term. The inhibitor of the invention also may be designed by utilizing known Virtual screening technology, comprising analyzing the structure of said stress protein, synthesizing suitable inhibitor in cyber space on computer, assaying efficacy of medicine by biosystem given on cyber and then deducing active substances expected and synthesizing them.

For the screening of the invention, chips that NSP protein, antibody and fragment thereof, DNA, RNA and fragment thereof are bound to solid support may be used. For the screening of the invention, protein array or nucleic acid array may be preferably used.

Especially, the screening method of the invention comprise treating host with stress such as virus, followed by applying candidate compounds, to determine whether the expression of the protein NSP from host is reduced or increase. Of course, the application of the candidate compounds may be executed when stress is added to host or before. Inhibitors may be used for preventing infectious diseases with pathogen. Specifically, the method for screening inhibitors of the invention may be accomplished by (a) assaying the expression level of NSP gene in cell infected with pathogen, (b) assaying the expression level of said NSP gene in animal cell infected with pathogen by applying tested substance to the same normal animal cell, followed by applying the same pathogen, (c) comparing (a) expression level of NSP gene assayed with (b) expression level of NSP gene assayed, and determining tested substance as inhibitor if (a) expression level of NSP gene assayed is higher than (b) expression level of NSP gene.



Furthermore, a particular embodiment of the invention provides a method for folding or regenerating protein, comprising treating the protein with protein denoted as SEQ ID NO: 1. A method that active regeneration of denatured protein is aided by utilizing protein having chaperone function or gene thereof, or folding of recombinant protein produced is aided by employing genetic recombinant method is well known(Cole, Structure 4:239-242 (1996)).

Also, in a particular embodiment, there is provided a composition for prevention of disease induced by expression of the stress protein, which comprises the nucleotide molecule encoding a polypeptide of the protein denoted as SEQ ID NO: 1.

In another aspect, the present invention provides vaccines for prevention and treatment of infectious disease induced by pathogens, which comprises a immunologically effective amount of the protein NSP or polynucleotide molecule encoding the protein with a immunostimulant.

To utilized NSP protein as immunogen for vaccine, standard immunological techniques may be employed. Preferably, protein denoted as SEQ ID NO: 1 is used. Immunologically effective amount of stress protein used in the invention means the amount to induce immunoreactions of mammals effective to prevent or alleviate infection of mammals by viroous, bacterial or parasitic pathogen, when administered to person in need, and in case of man, the amount is about 2mg to 10mg.

Administration of the stress protein of the invention is accomplished by various standard procedures. For more detailed description of such procedures, literature Antibodies, A Laboratory Manual, Cold Spring HarborLaboratory, ed. E. Harlow and D. Lane(1988) is referred. If polypeptide is used, pharmaceutically acceptable adjuvant, for example, complete or incomplete Freund's adjuvant, RIBI (muramyl dipeptides) and

ISCOM (immunostimulating complexes) are preferably administered. Preferably, the composition may comprise water-in-oil type emulsion or aluminium hydroxide as adjuvant.

Such vaccine composition is administered to patient or treated animal once or more. When administered, most effective mode and dosage regimen depend on immunological level, particular agent and/or adjuvant used when applied, expected degree and course of infection, former treatment, health condition of patient or animal and reaction to immunotreatment etc., for example, immunocompetent patient, immunological polypeptide of most high degree, less dosage and necessary immunity treatment number. Similarly, dosage and applying time shall be lowered if polypeptide is administered as adjuvant.

A part of the stress protein of the invention may be used in form of pharmaceutically acceptable salt. Suitable acid and base that can make polypeptide of the invention into salt are known in the art, comprising inorganic and organic acid and base.

The invention also provides DNA immunizing vaccine against pathogens. DNA immunization method is distinguished from former immunization method that attenuates or utilizes killed pathogen or partial component of pathogen, from the point that DNA encoding particular component of pathogen is directly injected into human body to achieve immunization, and has advantages of removing the risk of infection that can occur in case of using live bacteria or dead bacteria because actual production of immunizing protein is achieved in host injected with DNA.

DNA immunization method injects directly DNA vaccine vector having gene encoding NSP protein antigen, into a living body, through the route of intramuscular or intradermal etc., and such DNA is expressed in transformed cell, being attached to Major histocompatibility complex I and II: MHC I and MHC II, to induce proper antibody and cell immunoreaction.

However, such DNA immunization method has a limitation that strong immunoreaction isn't induced to antigen having low immunocompetence because frequency of immunized protein to be expressed in living body is low. Therefore, to enhance the efficiency of DNA immunization method, adjuvant such as cytokine gene or costimulatory molecule gene necessary for the activation of immune cell may be used (Geissler, M. et al., J. Immunol., 159, 5107-5113(1997), Iwasaki, A. et al., J. Immunol., 158, 4591-4601(1997)).

In the Examples described below as examples of the invention, after infecting salmon cells with virus, the protein NSP was separated from the cells to prepare monoclonal antibodies against the protein NSP. As a result of immunoprecipitation by using antibody against said protein NSP, it was found that a protein of SEQ ID NO: 1, specific example of the invention, was a protein having molecular weight of 90kDa as FIG. 1.

In addition, other embodiment of the invention confirmed expression aspect of NSP gene, after infection of fish cell with virus, as shown in FIG. 2, in cell not infected with virus, expression of NSP gene wasn't recognized while, in cell infected with virus, increase in expression of NSP gene was observed 24 hrs after. As shown in FIG. 3, NSP protein denoted as SEQ ID NO: 1 showed high similarity with TraC that is amino acid sequence of oligopeptide-binding protein. Generally, oligopeptide-binding protein is known as protein with chaperone function. To confirm whether NSP protein of the invention does function as chaperone or not, as expected from the sequence, in one example of the invention, said NSP gene was excessively expressed in E. coli, and then activity of  $\alpha$ -glucosidase was measured.

For excessive expression of NSP gene, pET29b vector(Novagen) was used, which has advantage that protein was effectively purified because histidines were bound to the expressed protein terminal. Therefore, in examples as described below, to separate

expressed protein NSP, chromatography using Ni-NTA His-binding resin(Novagen) was employed. FIG. 4 illustrates that the separated protein is a protein NSP having 90kDa of molecular weight.

It has been known that chaperone protein such as GroEL, Hsp90 and Hsps were concerned in folding of  $\alpha$ -glucosidase (Buchner et al., J. Biol. Chem. 268:1517-1520, 1993; and Jakob et al., Nature, 358:169-170, 1992). Therefore, after denaturing such  $\alpha$ -glucosidase, protein NSP, DnaK and BSA as control were added to measure the degree of folding, as a result, as shown in FIG. 5a, it can be found that in case of addition of the protein NSP, folding activity of  $\alpha$ -glucosidase is high. Such result confirms that NSP protein of the present invention is a protein with activity as chaperone.

The forgoing embodiments are merely exemplary and are not to be construed as limiting the present invention. The present teachings can be readily applied to other types of apparatuses. The description of the present invention is intended to be illustrative, and not to limit the scope of the claims. Many alternatives, modifications, and variations will be apparent to those skilled in the art.

## Example 1

### Cell culture and virus purification

#### 1-1) Cell culture

A fish cell line, CHSE-214 (ATCC CRL, 1681), from chinook salmon embryo (Lannan et al., 1984) was grown at 18°C, as monolayers in Eagle's minimum essential medium, with Earle's salts supplemented with 10 % fetal bovine serum (Gibco, USA) and penicillin-streptomycin (50 IU/ml and 50  $\mu$ g/ml, respectively, Flow Laboratories).

## **1-2) Virus purification**

IHNV-PRT strain was isolated from whole fish homogenates of IHNV-infected fry of rainbow trout. IHNV-PRT is a Korean strain of IHNV (Park et al, Journal of Fish Diseases, 6, 19-25 (1993)) and was deposited for the purpose of patent procedure in Korean Collection for Type Cultures (KCTC) at Korea Research Institute of Bioscience and Biotechnology (deposit number, IHNV-PRT KCTC 18089P)

CHSE-214 cells were cultivated in 75 cm<sup>2</sup> culture flask (Corning) and inoculated with 0.01 M.O.I (multiplicity of infection) of IHNV-PRT. After extensive CPE appeared in the monolayer, the culture supernatant was collected and cellular debris was removed by centrifugation at 4000g for 10 min. Virus particles in the supernatant were precipitated with 7% (w/v) polyethyleneglycol (PEG-6000, Sigma, USA) and collected by centrifugation at 6000g for 30 min. The virus particles in the precipitates were resuspended in TNE (Tris 0.01M, pH8.0, NaCl 0.1M, EDTA 0.001M) and purified by discontinuous sucrose gradient (50, 35, and 20%) centrifugation at 80000g for 90 min followed by continuous sucrose gradient (5-30%) centrifugation at 50000g for 30 min in SW50.1 rotor (Beckman Instruments). The virus was concentrated by centrifugation at 115000g for 60 min and the resulting virus pellet was resuspended in TNE and stored at - 20°C

## **Example 2**

### **Preparation of monoclonal antibody**

#### **2-1) Immunization**

Balb/c mice were immunized intraperitoneally four times with IHNV-PRT purified by method described in Example 1. Mice were first injected intraperitoneally with 5 ug of IHNV-PRT mixed with Freund's complete adjuvant (Sigma, USA) and received additional



three times of intraperitoneal injection with 5 ug of IHNV-PRT mixed with Freund's incomplete adjuvant (Sigma, USA). Mice were received a final intravenous injection with 5 ug of IHNV-PRT 3-4 days before fusion.

## 2-2) Preparation of hybridoma

Spleens were collected from immunized mice and red blood cells in the spleen cells were destroyed by adding 0.83%  $\text{NH}_4\text{Cl}$  in HEPES (Sigma). Spleen cells were fused with SP-2/0 myeloma cells (10:1 ratio) in polyethylene glycol solution (45% PEG-1500, 5% DMSO, 50% serum-free RPMI). The hybridoma cells were resuspended in RPMI-1640 medium containing HAT (0.1 mM hypoxanthine,  $4 \times 10^{-4}$  mM aminopterin,  $1.6 \times 10^{-2}$  mM thymidine) and 10 % fetal bovine serum (Gibco, USA) and plated in 96-well culture plates containing macrophages as feeder cells. After incubation for 2 weeks, culture media was changed to RPMI-1640 containing HT (0.1 mM hypoxanthine and  $1.6 \times 10^{-2}$  mM thymidine) for 1 week and subsequently to RPMI-1640.

## 2-3) ELISA screening

Hybridomas secreting monoclonal antibodies against IHNV were screened using an ELISA. Microtiter plates (Nunc, Denmark) were coated with 1 ug protein/ml of purified IHNV in coating buffer (0.5 M carbonate-bicarbonate, pH 9.6) at  $4^\circ\text{C}$  for 16 h. The plates were blocked with 1 % bovine serum albumin in PBS for 1 h at room temperature. After washing, monoclonal antibodies appropriately diluted in PBS containing 0.05% Tween 20 were added, incubated for 2 h and washed three times with PBS-Tween 20 (0.05%). Alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma, USA) was added and was detected by addition of alkaline phosphatase substrate, p-nitrophenyl phosphate (1 mg/ml in

0.1 M NaHCO<sub>3</sub> and 1.0 mM MgCl<sub>2</sub>, pH 9.8). After incubation for 30 min, the reaction was stopped by adding 20 ul of 3 M NaOH. The absorbance was read at 405 nm.

#### 2-4) Subcloning by limiting dilution

5 Hybridomas selected by ELISA screening were subcloned by limiting dilution. Macrophage feeder cells were collected from ascites fluid of Balb/c mice and cultivated in 96-well culture plate. Hybridoma cells were diluted to final concentration of 100 cells/ml, 50 cells/ml, and 5 cells/ml and each diluted cells were inoculated into 96-well culture plate containing 1-day cultured macrophage feeder cells (10 cells/well, 5 cells/well, and 0.5  
10 cell/well, respectively). After incubation for 7 days, wells containing single colony were selected and re-screened by ELISA. This subcloning step was conducted twice and the selected clones were expended in culture flask for in vitro production of monoclonal antibodies. The culture supernatant containing monoclonal antibody was stored at -20°C. The clone was deposited for the purpose of patent procedure in Korean Collection for Type  
15 Cultures (KCTC) at Korea Research Institute of Bioscience and Biotechnology (deposit number, KCTC 10128BP).

Monoclonal antibodies raised against the NSP were selected by Western blotting using purified virion as antigens and monoclonal antibodies as antibodies, according to the methods of Towbin et al. (Towbin et al., Proc. Natl. Acad. Sci., 76, 4350-4354 (1979)).

#### 2-5) Preparation of monoclonal antibodies against recombinant NSP

cDNA corresponding to the NSP was subcloned into NcoI-XhoI site of a pET29b and E.coli BL21(DE3) cells were transformed with this clone. Recombinant NSP was expressed as His-tagged protein in E.coli and purified on Ni-NTA column. The purified

recombinant NSP was used as an antigen for the preparation of monoclonal antibodies against NSP. Balb/c mice were immunized intraperitoneally four times with purified recombinant NSP (20 ug per mice) mixed with Freund's adjuvant (Sigma, USA) and received an additional intravenous injection 3-4 days before fusion. Spleen cells from immunized mice were fused with SP-2/0 myeloma cells in 50% polyethylene glycol 1500 (Sigma, USA). The cells were resuspended in RPMI-1640 medium containing hypoxanthine, aminopterin, thymidine and 10% fetal bovine serum (Gibco, USA) and plated in 96-well culture plates. Hybridoma supernatants were screened using ELISA and neutralization test. Selected hybridomas were cloned a minimum of 2 times by limiting dilution on macrophage feeder cells and then expended in culture flask for in vitro production of monoclonal antibodies. The proteins recognized by the monoclonal antibodies were confirmed by Western blotting using purified recombinant protein as antigen. As enlisted in Table 1, five monoclonal antibodies were prepared and among them BB10 showed highest binding activity to the NSP.

**【Table 1】 ELISA titer of monoclonal antibodies against NSP**

Monoclonal antibodies against the NSP	ELISA (OD <sub>405</sub> )
AD2	0.792
BB10	1.721
BC9	0.893
BF1	0.749
BF11	0.654

### Example 3

#### Purification and identification of NSP

##### 3-1) Purification of NSP

5           The CHSE-214 cells were harvested 24 h after IHNVPRT infection. Cell lysates were prepared by using lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 1 % NP-40, 0.5% deoxycholate, 1 mM  $MgCl_2$ , 1 mM phenylmethylsulfonyl fluoride) and were preadsorbed with 10 ul of protein G-Agarose (50% suspension in lysis buffer) (GIBCO) for 2 h at 4°C. Preadsorbed supernatant (200 ul) was then mixed with 10 ul of  
10       monoclonal antibody (MAb) BB10 raised against the NSP (as described in Example 2) and incubated overnight at 4°C. The immunoprecipitates were washed three times by centrifugation at 10,000g for 10 sec.

##### 3-2) Identification of the NSP by Western blotting

15           Polypeptides in the immunoprecipitates were electrophoresed in a 10% separating gel under reducing conditions. Molecular masses were assessed in comparison to molecular massstandards (Bio-Rad, California, USA). The proteins on acrylamide gels were transferred to nitrocellulose membranes. Membranes were blocked with 2% bovine serum albumin and then incubated with the 5000-fold diluted monoclonal antibody BB10. Alkaline  
20       phosphatase-conjugated secondary antibodies-goat anti-mouse IgG (Sigma, USA) were added and incubated for 2 h at room temperature. After washing 5 times with PBS, color was developed by adding 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Sigma) in an alkaline phosphatase buffer (100 mM NaCl, 5 mM  $MgCl_2$ , 100 mM Tris, pH 9.8) for 15 min.

As shown in Fig. 1 (lane 1, purified NSP by immunoprecipitation; lane 2, crude cell lysate of CHSE-214), NSP protein was detected by coomassie staining and Western blotting.

#### **Example 4**

##### **Peptide sequencing of NSP**

To identify the N-terminal amino acid sequence of the obtained proteins, the NSP was immunoprecipitated using MAb BB10 and then electrophoretically separated by SDS-PAGE as described in Example 3. The protein band was excised and the protein was eluted using gel eluter (Hoeffer). Two purified samples were submitted for amino acid sequencing. One sample was electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane and submitted for N-terminal amino acid sequencing. The second sample, approximately 20 pmol of the heavy chain protein, was subjected to digestion with 5 units of Arg-C at 25°C, analyzed by SDS-PAGE, electrophoretically transferred to a PVDF membrane, and then submitted for internal protein sequencing to the Basic Science Research Center, Korea. Analysis of Arg-C digestion product resulted in the determination of the primary sequence of three peptides, NSP-5 (aa 481-493), NSP-7 (246-259), and NSP-9 (425-439).

#### **Example 5**

##### **The NSP cDNA isolation and cloning**

###### **5-1) Preparation of probe for NSP gene**

Six degenerate oligonucleotides were synthesized based on the amino acid sequence of three peptides and named as NSP-5F, NSP-5R, NSP-7F, NSP-7R, NSP-9F, and NSP-9R (sequence number 3-8). PCR was carried out using the primer sets 5F-7R, 5F-9R,



7F-5R, 7F-9R, 9F-5R, and 9F-7R. A unique 186-bp product (sequence number 9) was isolated from gene amplification reaction using primer sets 9F-5R (sequence number 7 and 4, respectively). The gene amplification reaction conditions were as follows: 1 cycle of 94°C for 5 min; 20 cycles of 92°C for 30 s, 60°C for 1 min with a 0.5°C decrease each cycle, and 72°C for 1 min; 20 cycles of 92°C for 30 s, 50°C for 1 min, and 72°C for 1 min; and 1 cycle of 72 °C for 5 min. The PCR product was cloned into pGEM-T vector (Promega), and sequenced using the chain-terminating, dideoxy method. Conceptual translation of the primary sequence verified that this amplified product contains the amino acids from peptides NSP-9 and NSP-5, including amino acids that were not utilized in the design of the oligonucleotid. The 186-bp product was labeled with [<sup>32</sup>P]dCTP by random prime reactions (Stratagen) and used to screen the cDNA library.

## 5-2) Construction of cDNA library and screening

The CHSE-214 cells were harvested 24 h after IHN-V-PRT infection. Total nucleic acids were extracted using the guanidium thiocyanate-acid phenol-chloroform method and mRNA was purified using oligo(dT)30-Latex suspension (QIAGEN Inc, USA). The cDNAs were synthesized using a cDNA cloning kit (Stratagene; ZAP-cDNA Synthesis kit). The cDNA was ligated into the EcoRI and XhoI site of lambda ZAP vector and packaged into phage lambda. The resultant phage lambda was infected into E. coli XL1-Blue MRF' strain. E. coli cells were infected according to the manufacturer's instruction and screened by hybridization with 32P-labelled probe described in Example 5-1.

Approximately 200,000 plaques were screened in duplicate, and 20 positive colonies were identified. Ten plaques were subjected to secondary screening, and two of these were purified by tertiary screening. The cDNA inserts were excised into pBlueScript, and

sequenced completely in both directions and contained a 1659-bp fragment corresponding to nucleotides 1086-2744.

### **5-3) 5'RACE (rapid amplification of cDNA ends) cloning of a full-length NSP**

5 To obtain a full-length cDNA sequence, 5' RACE amplification was carried out by using 5' RACE kit (GibcoBRL) according to the manufacturer's instruction. CHSE-214 cells were harvested 24 h after IHNIV infection, and total RNA was extracted. One ug of the total RNA was reverse transcribed using MMLV reverse transcriptase (GibcoBRL, USA) and the first gene-specific primer (sequence number 10). A poly(C) tail was added to the 3'-end of  
10 the cDNA using terminal transferase (GibcoBRL). The dC tailed cDNA was amplified by PCR with 5'RACE abridged anchor primer (sequence number 11) and the second gene-specific primer (sequence number 12). The PCR was allowed to proceed for 35 cycles of 94°C (1 min), 55°C (1 min) and 72°C (1 min). The PCR product was re-amplified by nested PCR with universal amplification primer (sequence number 14) and nested gene-specific primer (sequence number 13). The PCR reaction was performed by 35 cycles of  
15 94°C (1 min), 50°C (1 min) and 72°C (1 min). The 5'RACE amplification product was cloned into pGEM-T vector (Promega, USA) and nucleotide sequencing was performed on an automatic DNA sequencer (Applied Biosystems, Inc., USA) according to the dye terminator procedure. This yielded nucleotides 1-1085 in the composite sequence and  
20 included the ATG start codon. The 2193-bp open reading frame, nucleotides 273-2465, encodes a conceptual translation product of 730 amino acids with a calculated molecular mass of 79.84 kDa (sequence number 2) (Fig 2 & 3).

### **Example 6**

## Expression and purification of NSP

For the expression of the NSP in *E. coli*, a plasmid (pET29-NSP) was constructed by inserting the cDNA (sequence number 2) corresponding to the NSP into NcoI-XhoI site of a pET29b in the correct reading frame.

5 A forward PCR primer (NSP-NcoI-U) was designed to contain the first 20 bp of ORF of NSP and NcoI recognition sequence was incorporated at the 5' end of this primer. A reverse PCR primer (NSP-XhoI-D) was designed to contain 20 bp of the 3' end of ORF of NSP. The stop codon of NSP was deleted to make a continuous open reading frame with the N-terminus of His-tag and XhoI recognition sequence was incorporated to this reverse PCR  
10 primer. PCR was carried out using these primer sets and pfu polymerase (Gibco, USA). The amplified polymerase chain reaction products were digested with NcoI and XhoI and ligated into the NcoI-XhoI sites of the pET29b vector to produce pET29-NSP (Fig. 4).

*E. coli* BL21(DE3) cells were transformed with pET29-NSP using  $\text{CaCl}_2$  method. NSP was expressed as His-tagged protein in *E. coli* and purified on Ni-NTA His-bind resin,  
15 according to the instruction of manufacturer (Novagen). Transformed *E. coli* BL21(DE3) cells were cultivated in LB medium at 37°C. When the  $\text{OD}_{600}$  reached 0.6, IPTG was added to a final concentration of 0.5 mM and cells were further cultivated for 4 h. Cells were harvested by centrifugation at 4000g for 15 min and resuspended in lysis buffer (50 mM  $\text{NaH}_2\text{PO}_4$ , 300 mM NaCl, 10 mM imidazole, pH 8.0). Cells were treated with lysozyme  
20 (final concentration, 1mg/ml) and destroyed by sonication. Cell lysate was centrifuged at 10,000g for 30 min and the resultant supernatant was loaded onto Ni-NTA spin column. Ni-NTA spin column was washed twice by centrifugation at 700g for 2 min with wash buffer. The recombinant protein was eluted from the Ni-NTA spin column with elution buffer by centrifugation at 700g for 2 min. The purified recombinant protein was recognized by

monoclonal antibody BB10 raised against the NSP and its band was a little shifted up because of the His-Tag (Fig. 5. Lane 1, purified recombinant NSP; lane 2, purified NSP from CHSE-214 cells by immunoprecipitation)

5

### Example 7

#### Increased expression of the NSP in fish cells by IHNV and IPNV infection

Four fish cell lines, enlisted in Table 2, were infected with IPNV or IHNV and at the indicated times, cells were harvested and analyzed for the total protein, translation and transcription products of the NSP. For the analysis of total protein,  $2 \times 10^6$  cells were analyzed on 10% SDS-PAGE gel under reducing condition. Polypeptides on the gels were visualized by coomassie staining or Western blotting using anti-NSP monoclonal antibody, BB10. For the analysis of translation product, cells were pulse-labeled for 30 min at the indicated times by adding 50 uCi of [ $^{35}$ S]methionine-cysteine (Amersham) per 25 cm<sup>2</sup> culture flask and the amount of newly-synthesized NSP was analyzed by immunoprecipitation using monoclonal antibody BB10. In order to analysis the transcription of the NSP gene, the amount of mRNA was measured at various times after virus infection by Northern blotting of RNA (Fig. 6). Total RNA was extracted from mock- and virus-infected cells and RNA samples (20 ug) were transferred to nylon membranes (Amersham; Hybond-N). Hybridization was done with [ $^{32}$ P]CTP-labeled probes prepared by random oligonucleotide priming method. The probe used was the cDNA clone of the NSP. As shown in Table 2, the NSP was expressed at a low level in a normal cells of four fish cell lines throughout the incubation period of 30 h. However, both in IHNV- and IPNV-infected cells, the expression of the NSP was prominently increased about 3 fold increase in total protein, 20 fold increase

20

in translation products, and more than 30 fold increase in transcription products at 30 h p.i.

The results indicate that the NSP is present in four different species of fish, salmon, trout, carp and minnow and the expression of the NSP is increased in all these cells by IPNV and IHNV infection. The results also possibly suggest that most species of fish possess the NSP and show increased expression of the NSP by virus infection.

**【Table 2】**

Increased expression of NSP in various kinds of fish cells by IPNV and IHNV infection

Cell line (origin)	virus	Time p.i. (hour)	Percent control*		
			Total protein	Translation product	Transcription product
CHSE-214 (Chinook salmon)	Mock	0	100	100	100
		10	108(±3.2)	112(±2.6)	98(±3.9)
		20	110(±4.7)	120(±2.9)	102(±2.4)
		30	104(±2.8)	119(±3.5)	119(±4.7)
	IPNV	0	98(±5.1)	120(±1.9)	109(±2.3)
		10	150(±2.9)	450(±7.8)	730(±8.1)
		20	300(±3.5)	1560(±14.9)	2790(±24.7)
		30	340(±5.5)	2080(±23.2)	3790(±27.8)
	IHNV	0	105(±2.4)	107(±4.2)	115(±4.9)
		10	220(±4.2)	540(±2.7)	690(±9.6)
		20	340(±4.9)	1870(±17.5)	3097(±33.5)
		30	380(±7.1)	2250(±19.3)	4042(±28.1)
FHM (Fethead minnow)	Mock	30	100	100	100
	IPNV	30	320(±2.9)	2210(±17.4)	3400(±42.3)
	IHNV	30	402(±4.5)	2640(±23.1)	4100(±3.78)
RTG-2 (Rainbow trout)	Mock	30	100	100	100
	IPNV	30	370(±3.8)	1900(±21.7)	3090(±39.1)
	IHNV	30	395(±5.1)	2450(±32.6)	3650(±36.7)
EPC (Carp)	Mock	30	100	100	100
	IPNV	30	340(±4.2)	2160(±25.6)	3280(±57.9)
	IHNV	30	385(±5.3)	2600(±38.4)	3980(±42.4)

\*mean. ±, standard error of mean (n = 3 experiments)

### Example 8

#### Chaperone activity of the NSP



We investigated whether the NSP acts as a molecular chaperone in the folding of proteins. Alpha glucosidase and alcohol dehydrogenase (ADH) were chosen as substrate for this reaction. Alpha glucosidase (Sigma) was denatured at a concentration of 15 M in 8 M urea, 0.1 M potassium phosphate, 2 mM EDTA, 20 mM dithiothreitol, pH 7.0, at 20°C. Renaturation was initiated by a 50-fold dilution in 40 mM Hepes-KOH, pH 7.5, at 20°C in the absence of additional protein or in the presence of various concentration of NSP, 5 M DnaK (StressGen), 20 M bovine serum albumin (Sigma). The enzymatic activity of alpha glucosidase was measured as described by Jakob et al. (Jakob et al., J. Biol. Chem., 268, 1517-1520 (1993)). Under our experimental conditions, the refolding yield of 0.3 M alpha glucosidase was increased from 4%, in the absence of added proteins, to 48% in the presence of 1 M NSP, and 16%, in the presence of 5 M DnaK. The addition of 20 M BSA did not affect the refolding of alpha glucosidase (Fig. 7A). Alcohol dehydrogenase (ADH) has a characteristic of autoaggregation at 37°C. However, the aggregation of the ADH was protected by addition of the NSP (Fig. 7B). The protection of ADH aggregation was dependent on the concentrations of added NSP and 53% and 84% protections of the ADH aggregation were attained in the presence of 0.5 M and 1.0 M NSP, respectively. These results suggest that, like molecular chaperones, the NSP interact with unfolded proteins, and increase their productive folding.

## Example 9

### Induction of antibody against the NSP in rainbow trout by infection of IPNV and IHNV

Sera were collected from normal, IPNV-infected and IHNV-infected rainbow trout with weight of about 5g, 100g, and 1kg and were assayed for the presence of antibodies

against the NSP by ELISA. ELISA plates were coated with 5 ug protein/ml of the NSP in coating buffer (0.5 M carbonate-bicarbonate, pH 9.6) for 1 h at room temperature. After blocking with 1% BSA, 2-fold serially diluted rainbow trout sera were added for 1 h at room temperature. Equal amount of phosphate buffered saline (PBS) was added as control. Rabbit anti-rainbow trout IgM and alkaline phosphatase conjugated goat anti-rabbit IgG were added sequentially. The bound alkaline phosphatase was detected by addition of p-nitrophenyl phosphate (1 mg/ml in 0.1M NaHCO<sub>3</sub> and 1.0 mM MgCl<sub>2</sub>, pH 9.8). The ELISA titer was expressed as the reciprocal of the highest dilution of sera showing the OD<sub>405</sub> value more than twice of the control. The results in Table 3 indicate that the titer of anti-NSP antibody is extremely low but increased significantly after IPNV or IHNV infection regardless of the body weight. This also suggests that IPNV and IHNV infection can be screened by measuring the level of NSP in the sera using ELISA.

【Table 3】

ELISA titer of anti-NSP antibodies in the sera of rainbow trout infected with IPNV and IHNV

Rainbow trout	weight	ELISA titer*
Normal rainbow trout	5g	16(±2.0)
	100g	8(±2.0)
	1kg	16(±2.0)
IPNV-infected rainbow trout	5g	1024(±16.0)
	100g	2048(±16.0)
	1kg	2048(±320.0)
IHNV-infected rainbow trout	5g	1024(±8.0)
	100g	4096(±64.0)
	1kg	2048(±32.0)

\*mean. ±, standard error of mean (n = 4 experiments)

### Example 10

**Neutralization activity of anti-NSP monoclonal antibody against IPNV and IHNV infectivity.**

100 TCID<sub>50</sub> of virus was incubated with 2-fold serially diluted antibodies. For this  
5 experiment, three different antibodies were used; monoclonal antibody BB10, rabbit anti-IHNV G antibody, rabbit anti-IPNV-VP2 antibody. The ability of antibodies to neutralize virus infectivity in vitro was determined by neutralization assay similar to that described by Okamoto et al. (Okamoto et al., Journal of Fish Diseases, 6, 19-25 1983)). Anti-IHNV G antibody was obtained from sera of rabbit immunized with recombinant IHNV G protein.  
10 Recombinant IHNV G protein was prepared by subcloning of IHNV G cDNA into pET29b vector, expression in E. coli BL21(DE3), and purification on Ni-NTA His-binding resin (Novagen). Anti-IPNV-VP2 antibody was obtained from sera of rabbit immunized with purified IPNV-VP2 according to methods described by Park & Jeong (Park and Jeong, Fish & Shellfish Immunology 6, 207-219 (1996)). IPNV virion was purified by  
15 ultracentrifugation on CsCl step gradient and separated on SDS-PAGE. IPNV-VP2 in the gel was eluted by electroelution. Neutralization titres of these antibodies are shown in Table 4. The two anti-IHNV G and anti-IPNV-VP2 antibodies possessed neutralizing activities at titres of 512 and 1024, respectively. The anti-NSP monoclonal antibody BB10 showed neutralizing activities at titres of 128 and 256 against IHNV and IPNV, respectively. This  
20 indicates that anti-NSP antibody has protective role against IPNV and IHNV infection. This also suggest that the NSP can be possible candidate for the vaccine against IPNV and IHNV infection.

【Table 4】

Neutralizing titer of anti-NSP antibody against IPNV and IHNV

Antibodies	Neutralization titer (ND <sub>50</sub> ) against virus	
	IPNV	IHNV
Anti-IPNV-VP2	1024	8
Anti-IHNV-G	16	512
Anti-NSP	256	128

**Example 11**5      **Screening for substance suppressing the expression of the NSP**

3 x 10<sup>5</sup> CHSE-214 cells in 24-well plate were treated for 12 h with various kinds of substance, enlisted in Table 5. After then cells were harvested and lysed by using 200 ul of lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 1 % NP-40, 0.5 % deoxycholate, 1 mM MgCl<sub>2</sub>, 1 mM phenylmethylsulfonyl fluoride). The amount of the NSP in the cell lysates were assayed by sandwich ELISA using polyclonal and monoclonal antibodies against the NSP. The ELISA plate (Nunc) was coated with rabbit anti-NSP polyclonal antibody and incubated with 200 l cell lysates. Monoclonal antibody BB10 and alkaline phosphatase conjugated anti mouse IgG (Sigma) was incubated and color was developed by adding p-nitrophenyl phosphate. The result in Table 5 showed that addition of ascorbic acid, NOS inhibitor suppressed the expression of the NSP significantly.

【Table 5】

Screening for substances suppressing the expression of the NSP

Substance	concentration	ELISA titer
None		0.135±0.014
IHNV infection	5moi	0.524±0.032
Ascorbic acid	1mM	0.118±0.009
	5mM	0.031±0.009
NaCl	1%	0.138±0.018
	2%	0.214±0.023
Copper sulfate	0.1mM	0.152±0.015
	0.5mM	0.421±0.037

NOS inhibitor (L-NAME)	0.1mM	0.118±0.012
	1mM	0.038±0.008
D-NAME	0.1mM	0.134±0.021
	1mM	0.129±0.018
NO generator (Sodium purssia)	100μM	0.156±0.013
	1mM	0.358±0.037
Cadmium sulfate	0.01mM	0.142±0.019
	0.1mM	0.413±0.023
2-mercaptoethanol	3mM	0.145±0.018
	30mM	0.359±0.034

### Example 12

#### Effect of ascorbic acid on the expression of the NSP and the growth of IPNV and IHNV

CHSE-214 cells were treated with various concentration of ascorbic acid from 0 to 5 mM 10 min prior to infection. After then the cells were inoculated and incubated with IHNV and IPNV for 1 h. At 30 h p.i., cells were harvested and analyzed for the NSP by Western blotting using monoclonal antibody BB10 and culture supernatant were collected and assayed for the titer of IHNV and IPNV. The results summarized in Figure 8 and Table 6 indicate that the expression of the NSP can be reduced by treatment with ascorbic acid in dose-dependent manner. With the decrease of the NSP, the growths of IHNV and IPNV were suppressed. This indicates that ascorbic acid can be used for therapeutics against IPNV and IHNV infection.

【Table 6】

Virus	Virus titer (log <sub>10</sub> ) in the presence of ascorbic acid (mM)			
	0	1	3	5
IPNV	5.9	4.5	3.8	3.5
IHNV	4.7	3.2	2.1	2.0

### Example 13

#### Effect of NOS inhibitor on the expression of the NSP and the growth of IPNV and



**IHNV**

CHSE-214 cells were treated with various concentration of NOS inhibitor, L-NAME, from 0 to 3 mM 10 min prior to infection. After then the cells were inoculated and incubated with IHNV and IPNV for 1 h. At 30 h p.i., cells were harvested and analyzed for the NSP by Western blotting using monoclonal antibody BB10 and culture supernatant were collected and assayed for the titer of IHNV and IPNV. The results summarized in Figure 9 and Table 7 indicate that the expression of the NSP can be reduced by treatment with L-NAME in dose-dependent manner. With the decrease of the NSP, the growths of IHNV and IPNV were suppressed. This indicates that NOS inhibitor such as L-NAME can be used for therapeutics against IPNV and IHNV infection.

**【Table 7】**

Virus	Virus titer (log <sub>10</sub> ) in the presence of L-NAME(mM)			
	0	0.5	1	3
IPNV	6.1	5.0	3.2	3.2
IHNV	4.4	3.1	2.2	2.0

**Example 14**

**Effect of antisense oligomer of the NSP on the expression of the NSP and the growth of IPNV and IHNV**

Based on the nucleotide sequence of the cDNA clone of the NSP, antisense oligomer (nucleotide -10 +10 5'-tggtccaagaatggcagctg-3') was designed. A scrambled oligomer with the same base composition (5'-acgtgatcgtagcatcgagt-3') was used as a negative control. 1 x 10<sup>6</sup> CHSE-214 cells were inoculated with IHNV or IPNV at one moi for 1 h. After washing out unbounded virus three times with EMEM, cells were treated with

antisense oligomer or scrambled oligomer at concentration of 0, 10 and 20 M in two ml of EMEM. At 30 h p.i., cells were harvested and analyzed for the NSP by Western blotting using monoclonal antibody BB10 and culture supernatant were collected and assayed for the titer of IHNV and IPNV. The results summarized in Figure 10 indicate that the expression of the NSP can be reduced by treatment with antisense oligomer in dose-dependent manner. With the decrease of the NSP, the growths of IHNV and IPNV were suppressed. This indicates that antisense oligomer can be used for therapeutics against IPNV and IHNV infection.

### Example 15

#### Induced expression of the NSP in human carcinoma tissues

The NSP or NSP homologous protein was found to be present in human cell as well as fish cells (Table 8). The expression of the NSP among human tumor tissues was determined by immunohistochemistry using monoclonal antibody BB10. A checkerboard multi-tumor block (DAKO Co.) was used for immunohistochemical analysis. The multi-tumor block was incubated with monoclonal antibody AB7, biotin conjugated anti mouse IgG and streptavidin conjugated peroxide. The antigen antibody reaction was visualized by adding AEC (3-amino 9-ethyl carbazole). The result in Table 8 showed significant immunostaining of the NSP in carcinomas such as colon cancer, breast cancer and stomach cancer but no apparent immunostaining in sarcomas. This suggest that the expression of the NSP is increased in human carcinomas such as colon cancer, breast cancer and stomach cancer tissues.

【Table 8】

NSP expression in human carcinoma tissues

Origin of tumor	Tumor	Number of sample	Number of positive	% positive
Epithelial origin	Breast cancer	4	4	100
	Colon cancer	2	2	100
	Prostate cancer	2	2	100
	Lung cancer	2	1	50
	Stomach cancer	2	1	50
	Renal cancer	2	1	50
	Mesothelioma cancer	2	1	50
	Thyroid cancer	2	0	50
	Ovary cancer	2	0	0
	Hepatocellular cancer	2	0	0
	Brain tumor	2	0	0
Mesenchymal origin	lymphoma	3	0	0
	Hodgkin's lymphoma	13	0	0
	Melanoma	4	0	0
	Sarcoma	10	0	0

**Example 16**

5

**Increased amount of NSP in the sera of human cancer patients**

Blood samples were collected from normal adult and carcinoma patients and were analyzed for the level of novel stress protein by sandwich ELISA using polyclonal and monoclonal antibody against the NSP. The ELISA plate(Nunc) was coated with rabbit anti-NSP polyclonal antibody and incubated with 5 ul sera of normal adults or carcinoma patients.

10

Monoclonal antibody BB10 and alkaline phosphatase conjugated anti-mouse IgG (Sigma) was incubated and color was developed by adding p-nitrophenyl phosphate. The result in Figure 11 showed significantly elevated level of NSP in sera from breast cancer and rectal cancer patients. This result suggests that carcinoma can be screened by measuring the level of NSP in the sera using sandwich ELISA.

15

**Example 17****Expression of NSP and NSP homologues in various kinds of cells**

In order to determine the presence of the NSP or the NSP homologues in a variety of animal cells other than fish cells, we used various kinds of cells derived from human, mouse, rat, hamster, monkey, fall armyworm, rice and prokaryotic bacteria as well as fish. Cell lysates were prepared from  $1 \times 10^7$  cells by using lysis buffer (10 mM Tris, pH 7.5, 10 mM NaCl, 0.1 mM EDTA, 1% NP-40, 0.5% deoxycholate, 1 mM  $MgCl_2$ , 1 mM phenylmethylsulfonyl fluoride) and the NSPs in the cell lysates were immunoprecipitated by adding 10  $\mu$ l of protein G-Agarose (from GIBCO) and monoclonal antibody BB10. The NSP in the immunoprecipitates were analyzed by Western blotting using monoclonal antibody BB10. The results in Table 9 describes that the expression of the NSP is not limited to fish cells. The NSP was detected in a variety of cells, including human lymphoma cells U-937, human carcinoma cells SNU-C2B, African green monkey kidney VERO, mouse embryo cells NIH/3T3, rat pituitary tumor cells GH3, Chinese hamster ovary CHO, and fall armyworm ovarian tissue Sf9. The mobilities of the NSP from these cells were the same as that from fish cell CHSE-214. These results showed that the presence of the NSP was widespread to a variety of cell types and species.

【Table 9】

Expression of NSP in various kinds of cells

Cell lines	Origin	Expression of NSP
CHSE-214	Salmon	+
RTG-2	Rainbow trout	+
FHM	Minnow	+
EPC	Carp	+
GF	Grunt	+
Jurkat	Human	+
U937	Human	+
SNU-C2B	Human	+
NIH/3T3	Mouse	+
GH3	Rat	+
CHO	Hamster	+
VERO	Monkey	+
BY-2	Tobacco	-
Embryonic primary cell line of rice	Rice	-
Sf9	Fall armyworm	+

**Example 18****5 Increased expression of NSP in colon tumors**

In order to examine the expression pattern of the NSP in colon tumors, immunohistochemical staining was conducted on 85 normal Normal Colonic Mucosa, 20 Colonic Adenoma and 65 Colonic Adenocarcinoma tissue samples using monoclonal antibody BB10. The tissue samples used in this experiment were collected from tumor patients and fixed with paraffin and treated with xylene and ethyl alcohol for rehydration. The tissue sections were treated with 10% goat serum for 20 min to prevent non-specific interaction of protein and treated with 3% hydrogenoxide for 15 min to destroy the activity of tissue peroxidase. After washing twice with distilled water and once with PBS for 5 min, the sections were incubated with monoclonal antibody BB10 for 2 h. After washing three times with PBS for 5 min, immunohistochemical staining was conducted using LSAB(labeled



streptavidin biotin) kit (DAKO, USA). The tissue sections were incubated with biotin conjugated anti mouse IgG and streptavidin conjugated peroxide. The antigen antibody reaction was visualized by adding AEC (3-amino 9-ethyl carbazole). After counter staining with meyer's hematoxylin tissue sections were observed with light microscope. In Scoring System, tissue sections with immunostained area above 10 % were decided to be positive and, among the positive, the tissue sections were divided into three score, 1, 2, and 3, based on the staining intensity and positive area proportion. The tissue sections with immunostained area below 10% were decided to be negative and grouped into 0 score. Correlation of Immunohistochemical expression was analysed by using SPSS version 10.0 statistically. The results were described in Table 10.

【Table 10】

Increased expression of NSP in colon tumor tissues

		Number of patients	Number of patients				P value
			0 score	1 score	2 score	3 score	
Normal		85	73	12	0	0	0.001
Adenoma		20	4	5	11	0	0.039
Adenocarcinoma		65	3	19	31	12	0.05
Sex	Male	31	1	8	16	6	0.07
	Female	34	2	10	16	6	
Duke's stage	A	7	0	4	1	2	0.15
	B	24	0	8	11	5	
	C	27	3	5	15	4	
	D	7	0	2	4	1	

The results showed that 14.1% of the normal tissues (12/85) was positive and all the positive samples were grouped into score 1 because of the low staining intensity. Of the 20 adenoma samples, 80% (16/20) was positive (5 grouped in score 1; 11 grouped in score 2).

In case of adenocarcinoma samples, 95.4% (62/65) was positive and the positive samples consisted of 19 samples in score 1, 31 samples in score 2, and 12 samples in score 3. This result suggests that the expression level of the NSP was extremely low in normal tissue but increased significantly in adenocarcinoma tissues. We also found that there is no difference in expression of the NSP between male and female of adenocarcinoma tissues. In addition, there was no significant difference among four Duke's stages of adenocarcinoma, A, B, C, and D.

In summary, the expression of the NSP is undetectable in normal colon tissue but increased with the progress to adenoma and adenocarcinoma, suggesting the possibility that the NSP plays a role in transforming the normal tissue to malignancy. However, among the adenocarcinoma, there was no difference in the expression of the NSP between male and female and among different stage of adenocarcinoma.

The collective results of the examples show that the NSP is a novel stress protein with chaperone activity and its expression is increased by infection of pathogen in various kinds of cells from all kinds of animal including human as well as fish. The NSP protein, antibody against the NSP, polynucleotide encoding the NSP, and inhibitor of NSP expression can be used to control infectious diseases in animals including human as therapeutic agents and vaccines and to diagnose infectious pathogens. Antibodies against the NSP and polynucleotide around the gene encoding the NSP can be used to screen any substances that regulate the expression of the NSP. In addition, antibodies against the NSP can be used to screen and diagnose various kinds of carcinoma.

## INDICATIONS RELATING TO DEPOSITED MICROORGANISM

## OR OTHER BIOLOGICAL MATERIAL

(PCT Rule 13bis)

A. The indications made below relate to the deposited microorganism or other biological material referred to in the description on page 54, line 10-20.	
B. IDENTIFICATION OF DEPOSIT <span style="float: right;">Further deposits are on an additional sheet <input type="checkbox"/></span>	
Name of depositary institution Korean Collection for Type Cultures(KCTC)	
Address of depositary institution( <i>including postal code and country</i> ) #52, Oun-dong, Yusong-ku, Taejon 305-333, Republic of Korea	
Date of deposit 26/11/2001	Accession Number KCTC 10128BP
C. ADDITIONAL INDICATIONS( <i>leave blank if not applicable</i> ) <span style="float: right;">This information is continued on an additional sheet <input type="checkbox"/></span>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE( <i>if the indications are not for all designated States</i> )	
E. SEPARATE FURNISHING OF INDICATIONS( <i>leave blank if not applicable</i> )	
The indications listed below will be submitted to the International Bureau later( <i>specify the general nature of the indications e.g., "Accession Number of Deposit"</i> )	

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Authorized officer

**What Is Claimed Is:**

1. A protein NSP with chaperone activity which is expressed in an increased level in animal cells stimulated by external stresses.

5 2. A protein which specifically binds to antibodies against the protein NSP with chaperone activity which is expressed in an increased level in animal cells stimulated by external stresses and has chaperone activities.

3. The protein according to claims 1 or 2, which corresponds to SEQ ID NO: 1.

10 4. The protein according to any one of claims 1 to 3, in which said external stress is selected from a group consisting of pathogenic infection, heat shock, nutrient deprivation, toxic metal, oxygen radicals, and metabolic disruption.

5. The protein according to claim 4, in which said pathogen is virus, bacteria or parasite.

15 6. The protein according to claim 5, in which said virus is selected from a group consisting of rhabdovirus, birnavirus, marine herpesvirus, channel catfish virus, marine nodavirus, iridovirus, infectious salmon anaemia virus, baculovirus, hepatopancreatic parvovirus, infectious hypodermal and haematopoietic necrosis virus, and yellow headed virus.

20 7. The protein according to claim 6, in which said rhabdovirus is selected from a group consisting of viral hemorrhagic septicemia virus(VHSV), infectious hematopoietic necrosis virus(IHNV) and spring viremia of carp virus(SVCV).

8. The protein according to claim 6, in which said birnavirus is infectious pancreatic necrosis virus(IPNV).

9. The protein according to claim 6, in which said marine nodavirus is selected from a group consisting of nervous necrosis virus and striped jack nervous necrosis virus.

10. The protein according to claim 6, in which said iridovirus is selected from a group consisting of fish lymphocyte disease virus and other marine iridovirus.

11. The protein according to claim 6, in which said baculovirus is selected from a group consisting of white spot syndrome baculovirus(WSBV), penaeus monodontype baculovirus(MBV), white spot red baculovirus and baculovirus penaei(BP).

12. The protein according to claim 5, in which said virus is selected from a group consisting of influenza, varicella, herpes simplex I, herpes simplex II, HIV-I, HIV-II, hepatitis A, hepatitis B, hepatitis C, adenovirus, measles and mumps.

13. The protein according to claim 5, in which said bacteria is selected from a group consisting of bacteria causing a disease selected from a group consisting of tuberculosis, gonorrhea, typhoid, meningitis, osteomyelitis, meningoccal septicemia, endometritis, conjunctivitis, peritonitis, pyelonephritis, pharyngitis, septic arthritis, cellulites, epiglottitis, salpingitis, otitis media, shigella dysentery and gastroenteritis.

14. The protein according to claim 5, in which said bacteria is selected from a group consisting of *Aeromonas hydrophila*, *Aeromonas salmonicida*, *Yersinia ruckeri*, *Pseudomonas aeruginosa*, *Pseudomonas anguilliseptica*, *Renibacterium salmoninarum*, *Vibrio anguillarum*, *Vibrio ordalii*, *Vibrio*, Pasteurellosis, Edwardsiellosis ictaluri, Edwardsiellosis tarda, *Cytophaga columnari*, and *Recherchia*.

15. The protein according to claim 5, in which said parasite is a marine parasite.

16. The protein according to any one of claims 1 through 15, in which said animal is



fish, domestic animals, and human.

17. The protein according to claim 16, in which said fish is aquacultured species.

18. The protein according to claim 17, in which said aquacultured species is selected from a group consisting of fin-fish, shellfish, flat-fish, seam bream and eel.

19. The protein according to claim 2, in which said antibody is monoclonal antibody.

20. The protein according to claim 2, in which said monoclonal antibody is BB10.

21. A process for producing the protein claimed in claims 1 through 3.

22. The process according to claim 21 characterized by comprising steps (a) inserting DNA sequence encoding the protein claimed in claims 1 through 3 into a vector having at least one of expression control sequences which is operatively linked to said DNA sequence and controls expression of said DNA sequence to form a recombinant expression vector, (b) transforming the recombinant expression vector into a host cell, (c) culturing the transformed cell in suitable media and conditions to express said DNA sequence, and (d) recovering substantially pure protein NSP from the culture, said protein NSP being expressed in increased amount in animal cells stimulated by external stresses and having chaperone activities.

23. The process according to claim 22, in which said DNA sequence is a nucleic molecule having the base sequence of SEQ ID NO: 2.

24. The process according to claim 22, in which said DNA sequence is a nucleic molecule having at least 80% identity to a nucleic molecule having the base sequence of SEQ ID NO: 2.

25. A nucleic molecule encoding the protein claimed in any one of claims 1 through 3.

26. The nucleic molecule according to claim 25, which has the base sequence of SEQ ID NO: 2.

5 27. The nucleic molecule according to claim 25, which has at least 80% identity to a nucleic molecule having the base sequence of SEQ ID NO: 2.

28. A recombinant expression vector including the nucleic molecule of claim 25.

10 29. The recombinant expression vector according to claim 28, which includes expression control sequence for said nucleic molecule.

30. A transformed or transfected cell with the recombinant expression vector of claim 28.

31. An antibody specifically binding to the protein of any one of claims 1 through 3.

15 32. The antibody according to claim 31, which is a monoclonal antibody.

33. The antibody according to claim 32, in which said monoclonal antibody is BB10.

34. A hybridoma producing the antibody of claim 31.

35. The hybridoma according to claim 34, which is KCTC 10128BP.

20 36. A process for producing the antibody of claim 31.

37. An inhibitor suppressing activity of the protein claimed in any one of claims 1 through 3.

38. An inhibitor against suppressing expression of the protein claimed in any one of claims 1 through 3.

39. The inhibitor according to claim 38, which is selected from a group consisting of antisense oligonucleotide, ascorbic acid and NOS gene inhibitor.

40. The inhibitor according to claim 39, in which said NOS gene inhibitor is N-nitro-L-arginine methyl ester hydrochloride(NAME).

5 41. An pharmaceutical composition for prevention or treatment of infectious disease in animals induced by pathogens, which comprises a prophylactically or therapeutically effective amount of the antibody of claim 31 and a pharmaceutically acceptable carrier.

10 42. The pharmaceutical composition according to claim 41, in which said disease in human is selected from a group consisting of tuberculosis, gonorrhea, typhoid, meningitis, osteomyelitis, meningoccal septiceia, endometritis, conjunctivitis, peritonitis, pyelonephritis, pharyngitis, septic arthritis, cellulites, epiglottitis, salpingitis, otitis media, sigella dysentery and gastroenteritis.

15 43. The pharmaceutical composition according to claim 41, in which said pathogen is virus, bacteria or parasite.

44. A process for prevention or treatment of infectious disease in animals induced by pathogens, which comprises using the pharmaceutical composition of claim 41.

20 45. An pharmaceutical composition for prevention or treatment of infectious disease in animals induced by pathogens, which comprises a prophylactically or therapeutically effective amount of the protein claimed in any one of claims 1 through 3 and a pharmaceutically acceptable carrier.

46. The pharmaceutical composition according to claim 45, in which said disease in human is selected from a group consisting of tuberculosis, gonorrhea, typhoid, meningitis, osteomyelitis, meningoccal septiceia, endometritis, conjunctivitis, peritonitis,

pyelonephritis, pharyngitis, septic arthritis, cellulites, epiglottitis, salpingitis, otitis media, sigella dysentery and gastroenteritis.

47. The pharmaceutical composition according to claim 45, in which said pathogen is virus, bacteria or parasite.

5 48. A process for prevention or treatment of infectious disease in animals induced by pathogens comprising using the pharmaceutical composition of claim 45.

49. An pharmaceutical composition for prevention or treatment of infectious disease in animals induced by pathogens, which comprises a prophylactically or therapeutically effective amount of the inhibitor suppressing activity of the protein claimed in  
10 any one of claims 1 through 3 and/or the inhibitor suppressing expression of the protein claimed in any one of claims 1 through 3, and a pharmaceutically acceptable carrier.

50. The pharmaceutical composition according to claim 49, in which said inhibitor is an antisense oligonucleotide.

51. The pharmaceutical composition according to claim 49, in which said  
15 inhibitor is ascorbinc acid or NOS inhibitor.

52. The pharmaceutical composition according to claim 51, in which said NOS inhibitor is NAME.

53. The pharmaceutical composition according to claim 49, in which said disease of human is selected from a group consisting of tuberculosis, gonorrhea, typhoid, meningitis, osteomyelitis, meningoccal septiceia, endometritis, conjunctivitis, peritonitis,  
20 pyelonephritis, pharyngitis, septic arthritis, cellulites, epiglottitis, salpingitis, otitis media, sigella dysentery and gastroenteritis.

54. The pharmaceutical composition according to claim 49, in which said pathogen is virus, bacteria or parasite.

55. A process for prevention or treatment of infectious disease in animals induced by pathogens, which comprises using the pharmaceutical composition of claim 49.

56. A vaccine for prevention or treatment of infectious disease in animals induced by pathogens, which comprises an immunologically effective amount of the protein claimed in any one of claims 1 through 3.

57. The vaccine according to claim 56, in which said disease of human is selected from a group consisting of tuberculosis, gonorrhea, typhoid, meningitis, osteomyelitis, meningoccal septiceia, endometritis, conjunctivitis, peritonitis, pyelonephritis, pharyngitis, septic arthritis, cellulites, epiglottitis, salpingitis, otitis media, sigella dysentery and gastroenteritis.

58. The vaccine according to claim 56, in which said pathogen is virus, bacteria or parasite.

59. A method for immunization of animals against pathogens, which comprises administering the vaccine of claim 57 to said animals.

60. A DNA vaccine for prevention or treatment of infectious disease in animals induced by pathogens comprising a sufficient amount of the nucleic molecule of claim 25 to induce immunity in said animals.

61. The DNA vaccine according to claim 60, in which said nucleic molecule is the polynucleotide denoted as SEQ ID NO: 2.

62. The DNA vaccine according to claim 60, in which said nucleic molecule has at least 80% identity to the polynucleotide denoted as SEQ ID NO: 2.

63. A method for immunization of animals against pathogens, which comprises administering the DNA vaccine of claim 60 to said animals.

64. A composition for diagnosis of disease accompanying with increase in the



expression level of the protein NSP, which comprises the antibody of claim 31.

65. The composition according to claim 64, in which said disease is cancer.

66. The composition according to claim 65, in which said cancer is large intestinal cancer, breast cancer, prostatic cancer, colon cancer, rectal cancer or stomach cancer.

67. A method for diagnosing disease accompanying with an increase in the expression level of the protein NSP, which comprises (a) contacting a biological sample of animal with the antibody of claim 31 and (b) detecting the presence of the conjugate of the protein NSP in the biological sample with the antibody.

68. The method according to claim 67, in which said biological sample is selected from a group consisting of tissue, cell, whole blood, bile, serum, sera, saliva, cerebrospinal fluid and urine.

69. A composition for diagnosis of disease accompanying with an increase in the expression level of the protein NSP, which comprises the polynucleotide encoding the protein of any one of claims 1 through 3.

70. The composition according to 69, in which said disease is cancer.

71. The composition according to claim 70, in which said cancer is large intestinal cancer, breast cancer, prostatic cancer, colon cancer, rectal cancer or stomach cancer.

72. A method for diagnosing disease accompanying with an increase in the expression level of the protein NSP, which comprises (a) reacting a biological sample with the polynucleotide coding for the protein of any one of claims 1 through 3 and (b) detecting the nucleic molecule in said sample which hybridizes with said polynucleotide.

73. The method according to claim 72, in which said biological sample is

selected from a group consisting of tissue, cell, whole blood, bile, serum, sera, saliva, cerebrospinal fluid and urine.

74. A method for diagnosing disease accompanying with increase expression of the protein NSP comprising (a) assaying the expression level of the NSP gene coding for the protein of any one of claims 1 through 3 in a biological animal sample and (b) comparing the assayed expression level of said NSP gene with a standard expression level of said NSP gene.

75. A method for screening inhibitors suppressing expression of NSP gene, which comprises (a) assaying the expression level of the NSP gene encoding the protein of any one of claims 1 through 3 in animal cells infected with a pathogen, (b) assaying the expression level of said NSP gene in the same normal animal cells treated with the substance to be tested and then infected with the same pathogen, and (c) comparing the expression level of the NSP gene assayed in (a) with the expression level of the NSP gene assayed in (b) and determining the substance as an inhibitor where the expression level of NSP gene assayed in (a) is higher than the expression level of NSP gene assayed in (b).

76. A protein array comprising the protein of any one of claims 1 through 3 or an active fragment thereof, and a solid support.

77. A protein array comprising the antibody of claim 31 or an active fragment thereof, and a solid support.

78. A nucleic acid array comprising the nucleic molecule of claim 25, and a solid support.

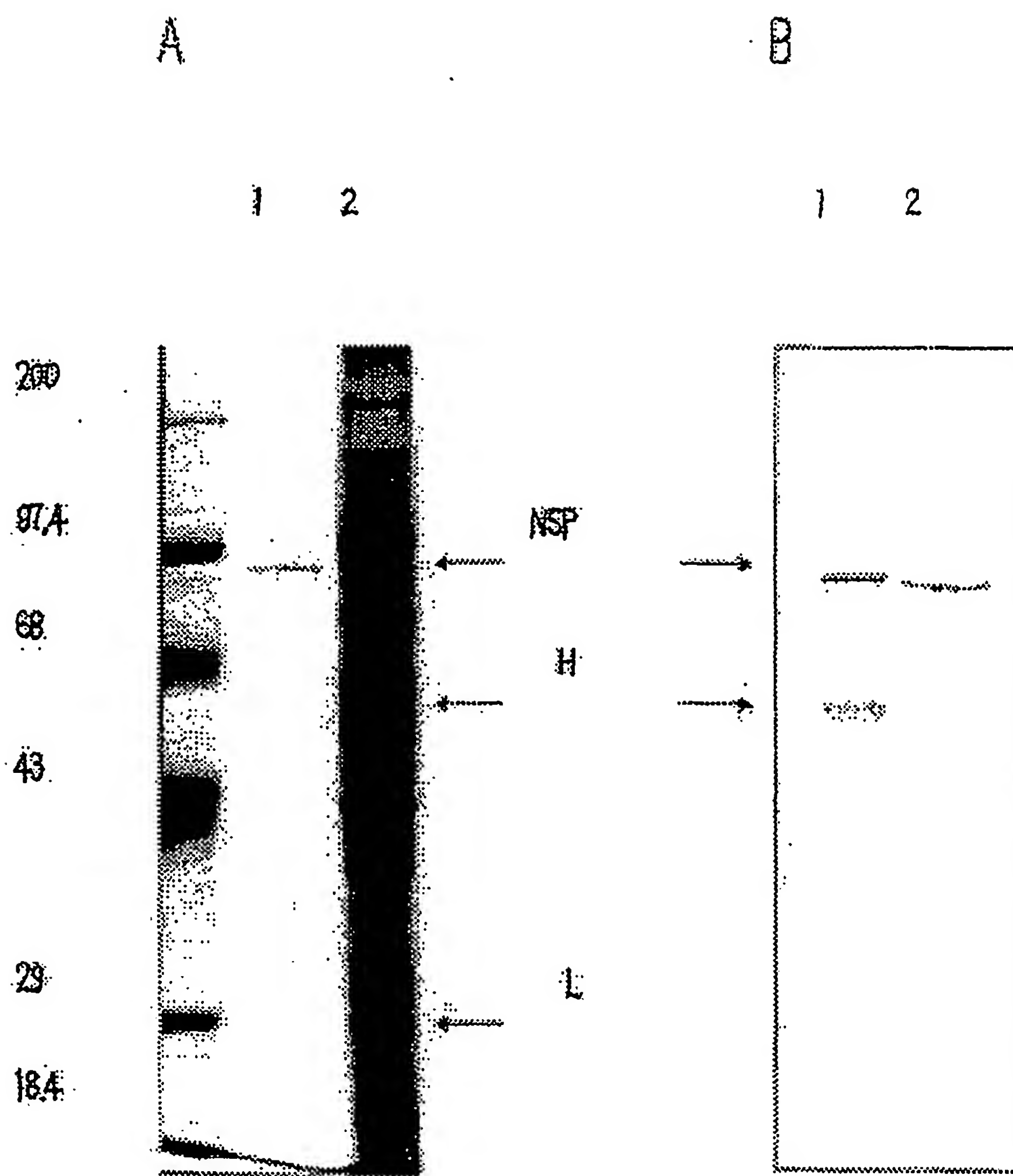
79. A method for screening inhibitors against activities of the stress protein of any one of claims 1 through 3 comprising (a) contacting said stress protein with candidate compounds, and (b) separating any compound suppressing chaperon activity of said stress

protein.

80. A method for folding or renaturation of a protein comprising treating the protein with the stress protein of any one of claims 1 through 3.

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FIG. 1



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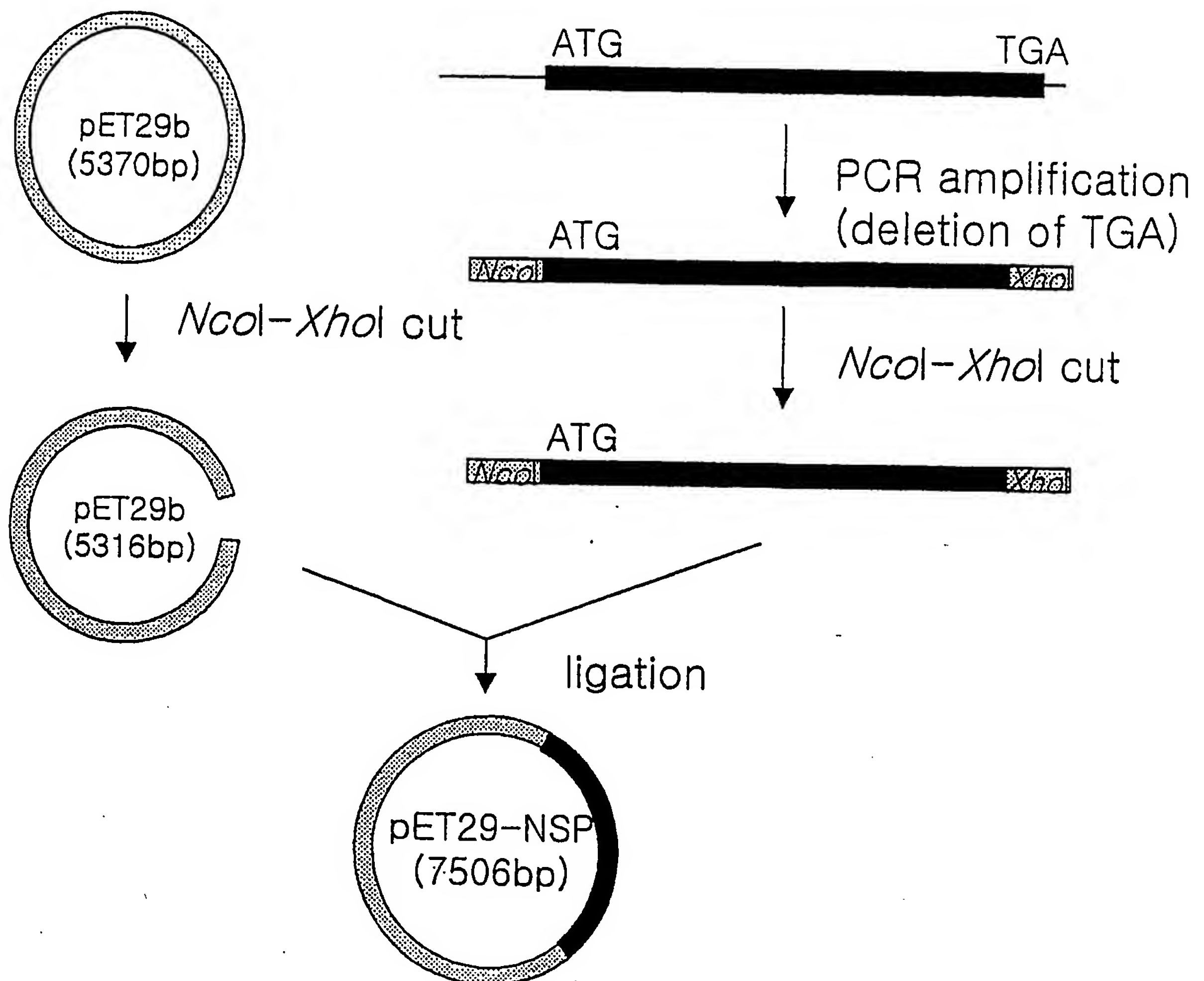
## FIG. 3

DppE-Bsub	1	-----MKRVKQLWGMGLALGLSFALMGCTANEQAGKEGSHDKAKTSGEKVLYVNNENEPTSFDPPIGFNNVSWQPLN
OppA-Bsub	1	-----MKQRWSIVTLMILFTLVLSACGFGGTGSNG-EGKKDSKGTTLNINIKTEPFSLEHPGLANDSVSGGVIR
OppA-Ecol	1	-----MTNITKRSIVAAGVLAALMAGNVALAADVPAG--VTLAEKQT--LVRNNGSEVQSILDPHKIEGVPEISNISR
TraC-Efae	1	-----MKKRTMLSFFCVSVLVLLGACNGKEGKNNNS--KSKEVEQVA--TFSAMTPVQSILDPATAVDQTSITLLA
NSP	1	MAAGEFVDVNGDGTWETLRLDLEKFDGTPINANTFDYSWRQLLDPDQLNVRASNLCDSSNLPLVNAEAYTKQKSQSD
DppE-Bsub	73	NIMEGLTRLGKDHEPEPAMAEKWSVS-----KDNKTYTFTIRENAKWT----
OppA-Bsub	69	QTFEGLTRINADGEPEEGMASKIETS-----KDGKTYTFTIRDGVKWS----
OppA-Ecol	68	DLFEGLLVSDLDGHPAPGVAESWDN-----KDAKVWTFHLRKDAKWS----
TraC-Efae	68	NVMEGLYRLDEKNQPPAIAAGQPKIS-----NNGKTYTIVIRDGAKWA----
NSP	81	DFNEPIWDAGDKGTIETANAFAFEDDGDFFHANVDGYFVYYYPSAAGTFGGNWDLLYGKGGGGYGGWVLQDAGVPTVL
DppE-Bsub	116	-NGDPVTAAGDFEYAWKRLDPKKGASSAFLGYF-----IEGGEAYNSGKGKKDDVKVTAKDDRTLEVTLEAP-QKYFLSV
OppA-Bsub	112	-NGDPVTAQDFEYAWKWLDPNNESQYAYQLYY-----IKGAEAAANTGKGSLLDDVAVKAVNDKTLKVELNNP-TPYFTEL
OppA-Ecol	110	-DGTFTVTAQDFVYSWQSRVDPNTASPYASYLOYGH---IAGIDEILEGKKPITDLGVKAIDDETLEVTLESEP-VPYFYKL
TraC-Efae	112	-DGTDTTADDFVTAWQRVDPKTASPSVELFSA-----IKNAKEIASGKQSKETLAVKSNGNKTIEIELEKP-TPYFTDL
NSP	161	PDGTFNPAAAGEKAKYQGAQPKLFYEDGTVVPVGEKGQPVGATAIPNDPVSWDKVGFKVVTGTETKFIITLTPKDAWYVKG
DppE-Bsub	189	VSNPAYFPVNEKVDKDNPKWFAES-----DTFVGNGPFLTEWKED---DSITMEKSDTYWDKDTVKLDKVKWAMVS
OppA-Bsub	185	TAFYTYMPINEKIAEKNKKWNTNAG-----DDYVSNGEPFMTAWKHS---GSITLEKNDQYWDKDKVKLKKIDMVMIN
OppA-Ecol	185	LVHPSTSPVPKAAIEKFGEKWTQP-----GNIVTNGAYTLKDWVNN---ERIVLERSPTYWNNAKTVINQVTYLPIA
TraC-Efae	185	LALTAYFPVQQAKEYGKDYGTSQ-----KSIVTNGAFNLTSLEGVGTSDKWTISKNKKEYWDQKEVSMEKINFQVVK
NSP	241	QLMSGITGVVHEAKYEAGKSADKRTTTYGTIDNPLVSYGAYNLIEWQDQ---VLXLFERNDKHYAAEYRIKIRYDVIA
DppE-Bsub	258	DRNTDYQMFQSGELDTA-----YVPAELSDQLLDQD--NVNIVDQAGLYFYRFNVNMEPFQEN-----IRKAFAMAV
OppA-Bsub	255	NNNTELLKQFQAGELDWAGMPLGQLPTESLPTLKKDG--SLEVEPIAGVYWKFNTEAKPLDNVN-----IRKALTYSL
OppA-Ecol	254	SEVTDVNRYRSGEIDMTN---NSMPIELFQKLKKEIPDEVHVDPLYCTYYEINNOKPPFNDVR-----VRTALKLGM
TraC-Efae	258	EINTGINLYNDGQLDDTP-----VTGEYAKQYKONKEFSTTLMANTMYLELNQREKNSILQLKN-----VRKAISYAI
NSP	318	DQSIADVNEFKAGRLLDIVG-----ASGEYFKEFKESP--QLKLTPTSTVFFRFAFNIDERPDGTTNPILGYKEFRQAFYYAI
DppE-Bsub	324	DQEEIVKYVTKNNEK-----TAHAFVSPGFTQPDGKDFREAGGDLIKPNESK-AKOLLEKGMKEE-----NYNK
OppA-Bsub	326	DRQSIKVNVTQGEQM-----PAMAAVPP--T---MKGFEDNKEGYFKDNDVKTAKEYLEKGLKEMGL--SKASD
OppA-Ecol	324	DRDIIVNKVKAQGNM-----PAYGYTPP-----YTDGAKLTQPEWFGWSQEK-RNEEAKLLAEAG---YTADK
TraC-Efae	326	DRSNYAKNILDNGSI-----SAVGVAQVAFDPSTKKDFANKMLVHFDTEK-AQSYWNKAKKELN-----IKE
NSP	391	DREEFASVVRAPAYANQGFIPVYIATEYGSVSYRGSAGKAVFAPLSPETTGFPNPTKAKQLFDAAYAKAVADNKISDGQ
DppE-Bsub	387	LPAITLTYSTKPEBKIAEAIQOKLKNLGL--VDVKLANMEWNVFLDQKALKFQFSQSSFLPDYADPISFLEAFQTGNS
OppA-Bsub	388	LPKIKLSYNTDDAHAKIAQAVQEMWKKNLG--VDVELDNSEWNVYIDKLHSQDYQIGRMGWLGDNDPINFLELFRDKNG
OppA-Ecol	384	PLTINLLYNTSDLEHKLALAAASSLWKKNLG--VNVKLVNQEWKTFDTRHQGTEDVARAGWCADYNEPTSFNTMLSNSS
TraC-Efae	389	QVTNLILTNEEETTKKAEYIQGQLEENLKG-LKITITPVPANVQIERVMKHDFITISLGGWQADYDPMSFLGNFESNSV
NSP	471	KVSVEYTYKVKETNDTVADWVKQTVFAIFGDKFELKLVSSDALDAWDNDFDMTFGGWQGLNFNFVSLMGQVYNSIS
DppE-Bsub	465	MN-----RTGWANKEYDQLIKQAKNEAD-----EKTRFSLMHQAEELLI
OppA-Bsub	466	GNN-----DTGWENPEFKLLNQSQTETD-----KTKRAELLKKAEGIFI
OppA-Ecol	462	MN-----TAHYKSPAEDSIMAETLKVTD-----EAORTALYTKAEQQLD
TraC-Efae	468	LN-----FGGYSNTKYDEYLLKDOTS-----DKRWKSLKKAELLIL
NSP	551	GAANMLENGFDTGNIQLSIELPETKAALQKVAEFNEETASEAQLAAYGRWTAALAKFGDSNTYSDTLDNVYKAAASTFY
DppE-Bsub	504	-----NEAPIIPVYFYNQVHLQNEQVKGIVRHFVGYIDLKWADKN-----
OppA-Bsub	506	-----DEMPVAPIYFYTDTWVQDENLKGIVMPGTGEVYFRNAYFK-----
OppA-Ecol	501	-----KDSAIVPVYVYVNRALVKPWVGGYTGDPLDNTYTRNMYIVKH-----
TraC-Efae	502	-----EDAGVVPFLQVGNKLLKSTMRNIETHSIGAKYDYKMKRLE-----
NSP	631	NVSDVNYAGRNDFFDRITAFAEALLDEMNOFSGASLLSDLCGAGSRSGLPSVLLWVGQTLTSLLLCLGAGVRWATCLE
DppE-Bsub		-----
OppA-Bsub		-----
OppA-Ecol		-----
TraC-Efae		-----
NSP	711	GGCLGGGLTPDPGLLDGYND



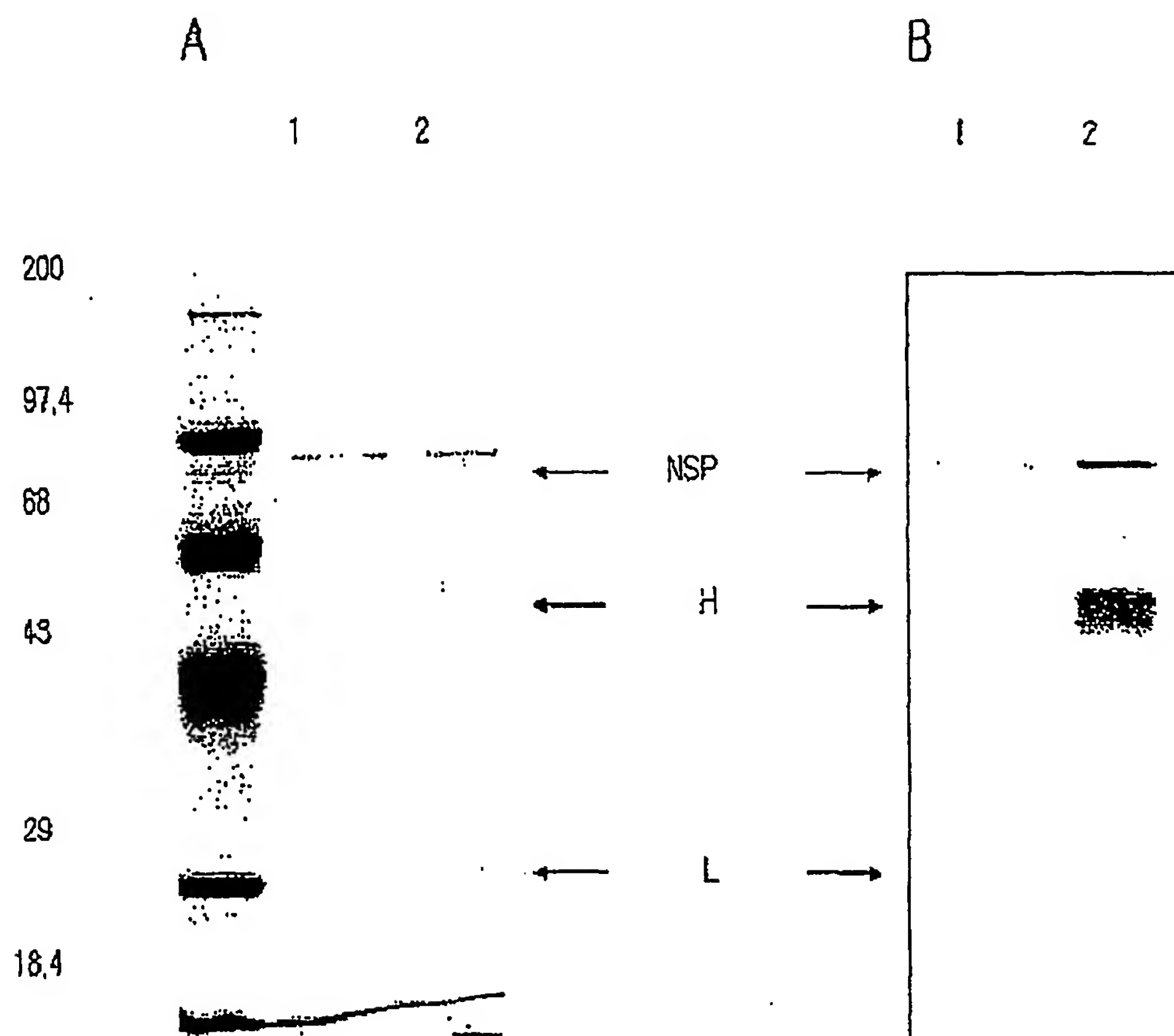
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FIG. 4



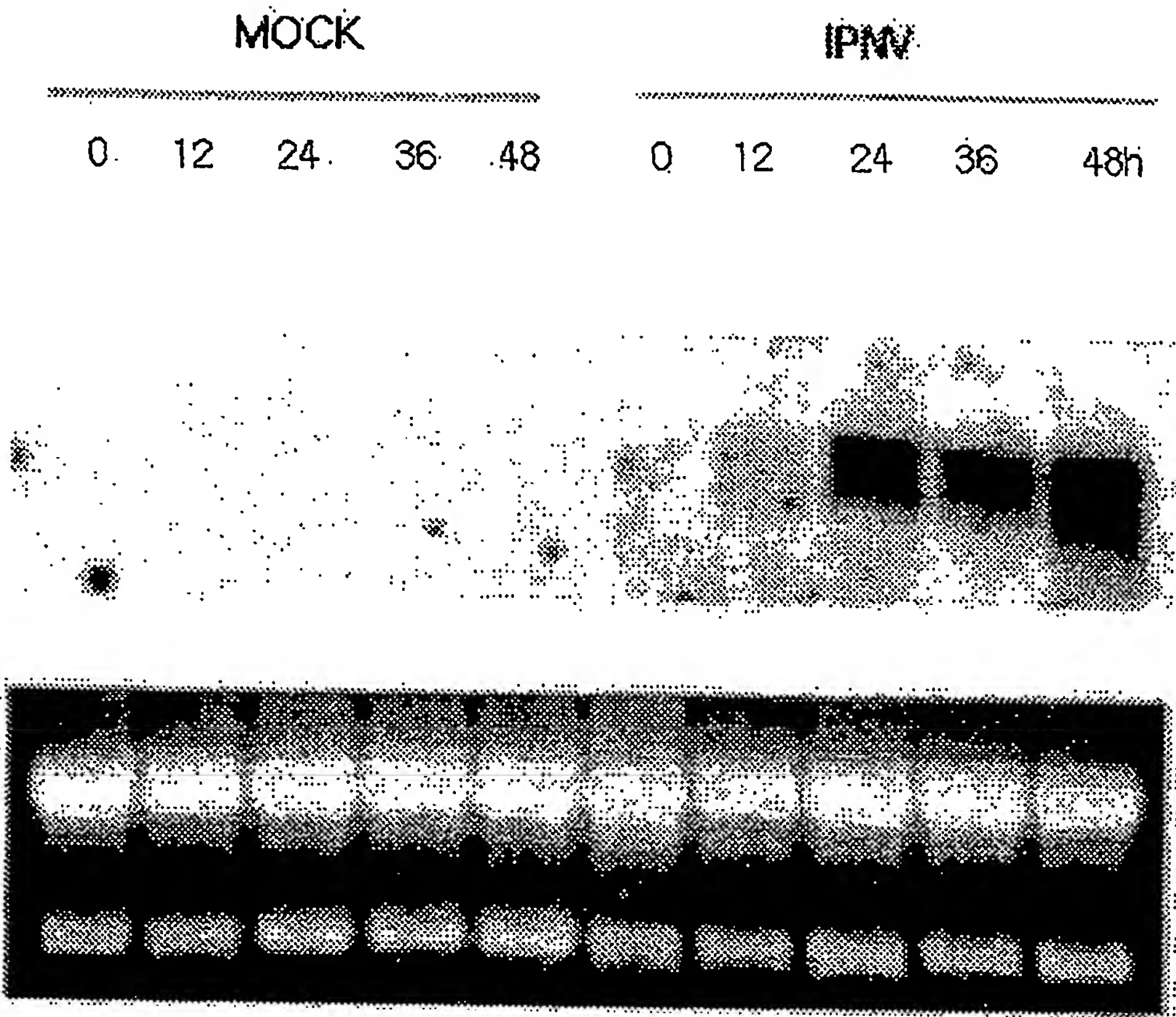
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**FIG. 5**



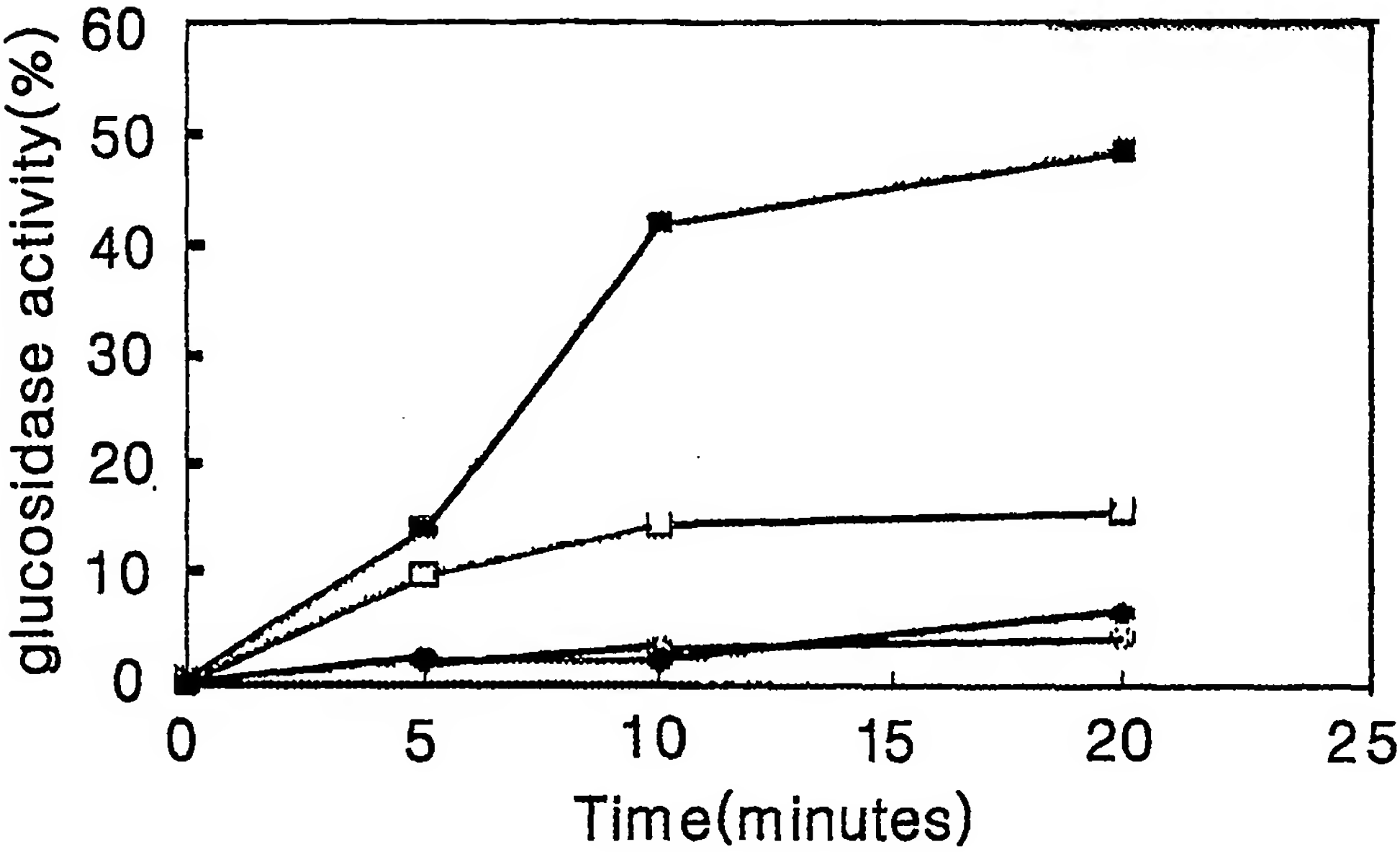
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FIG. 6



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FIG. 7A



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FIG. 7B

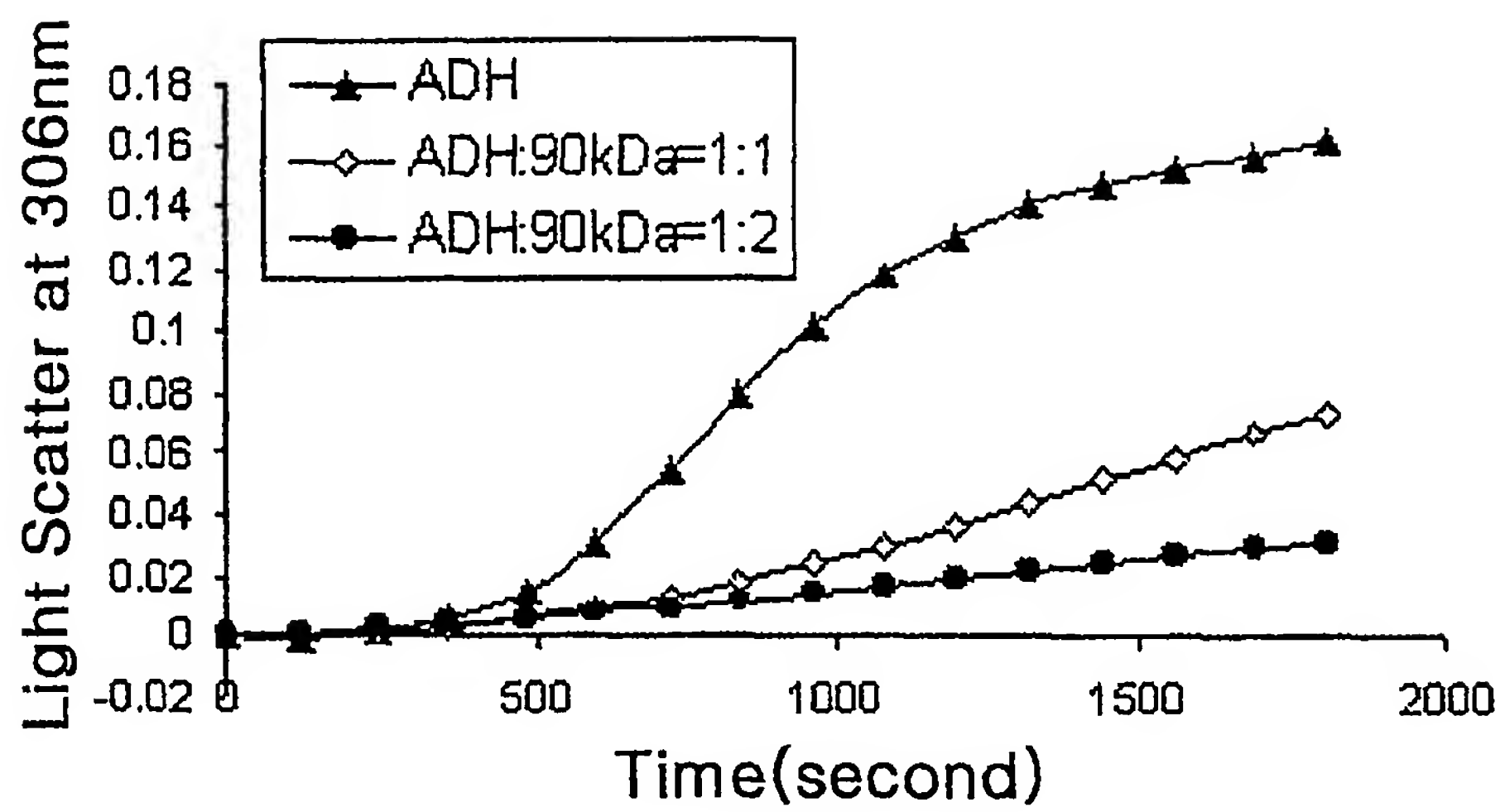
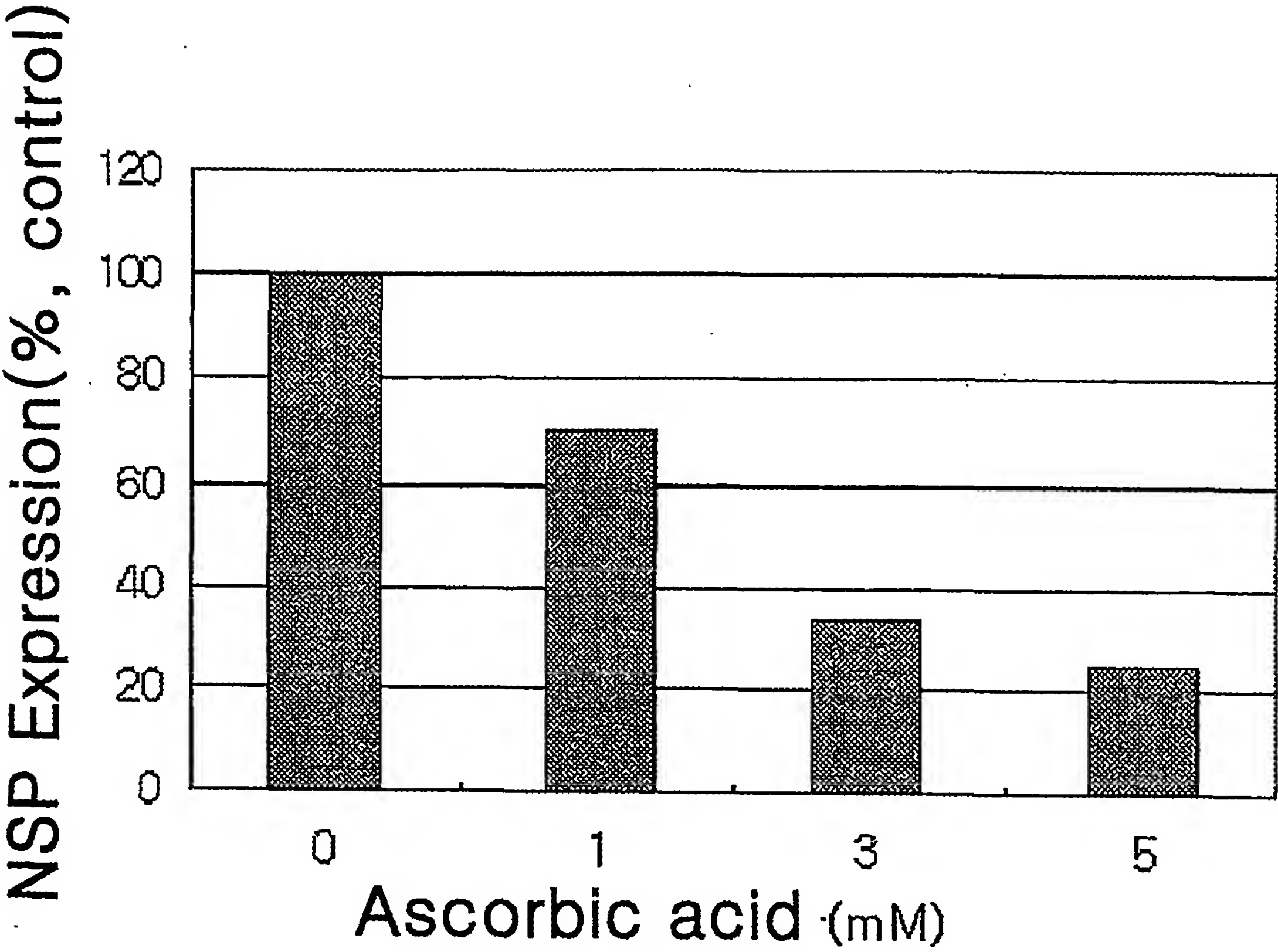
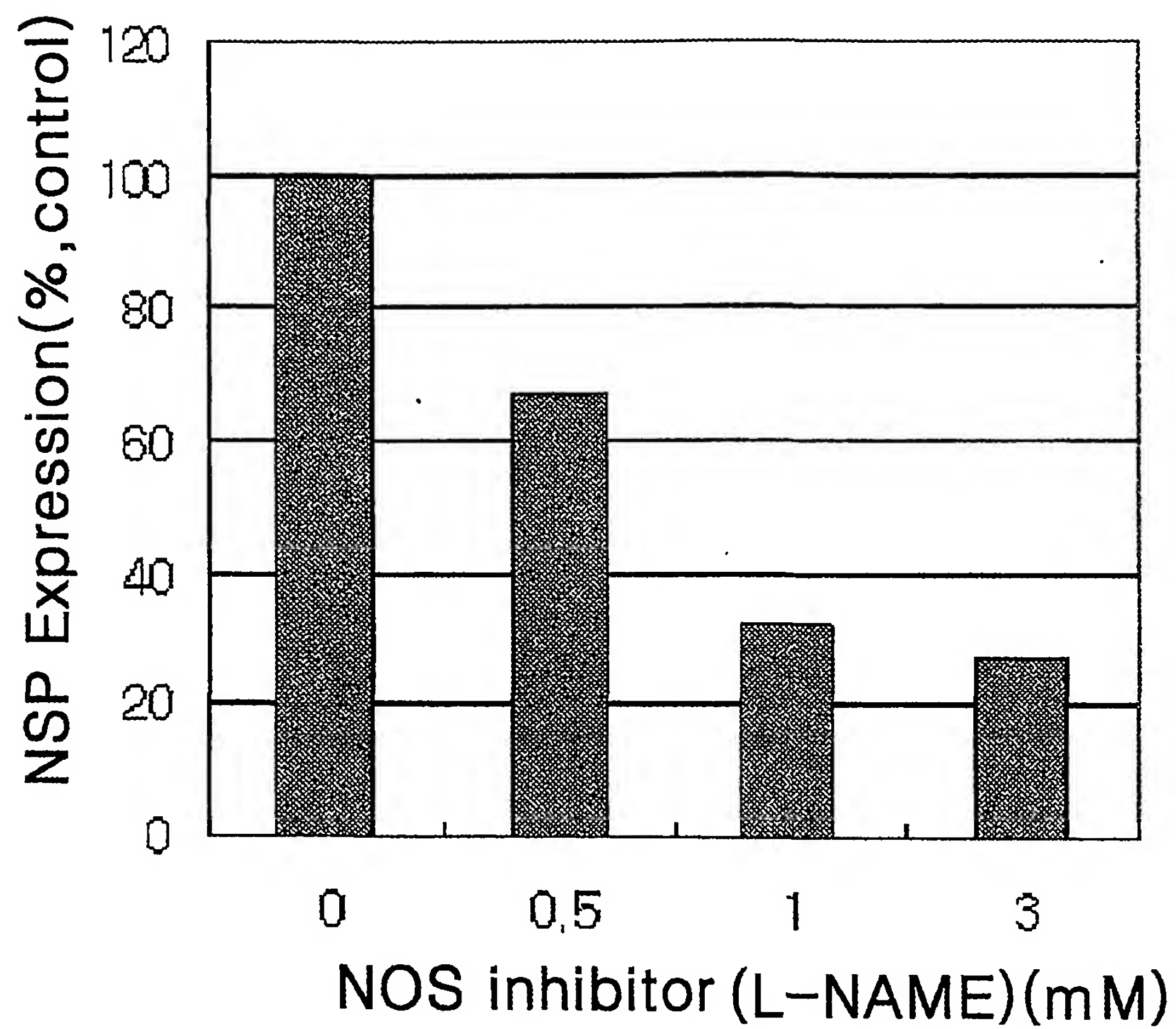




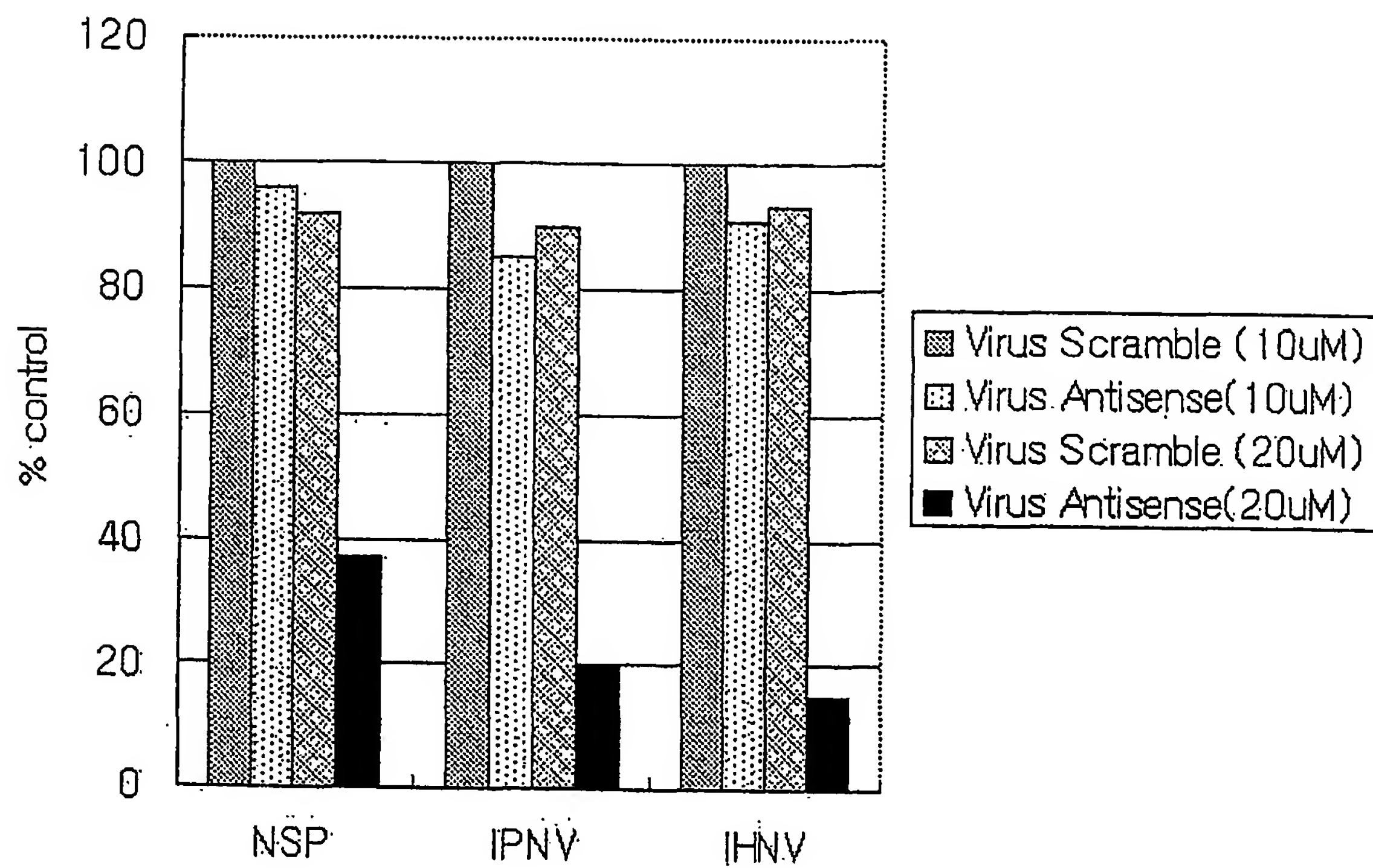
FIG. 8



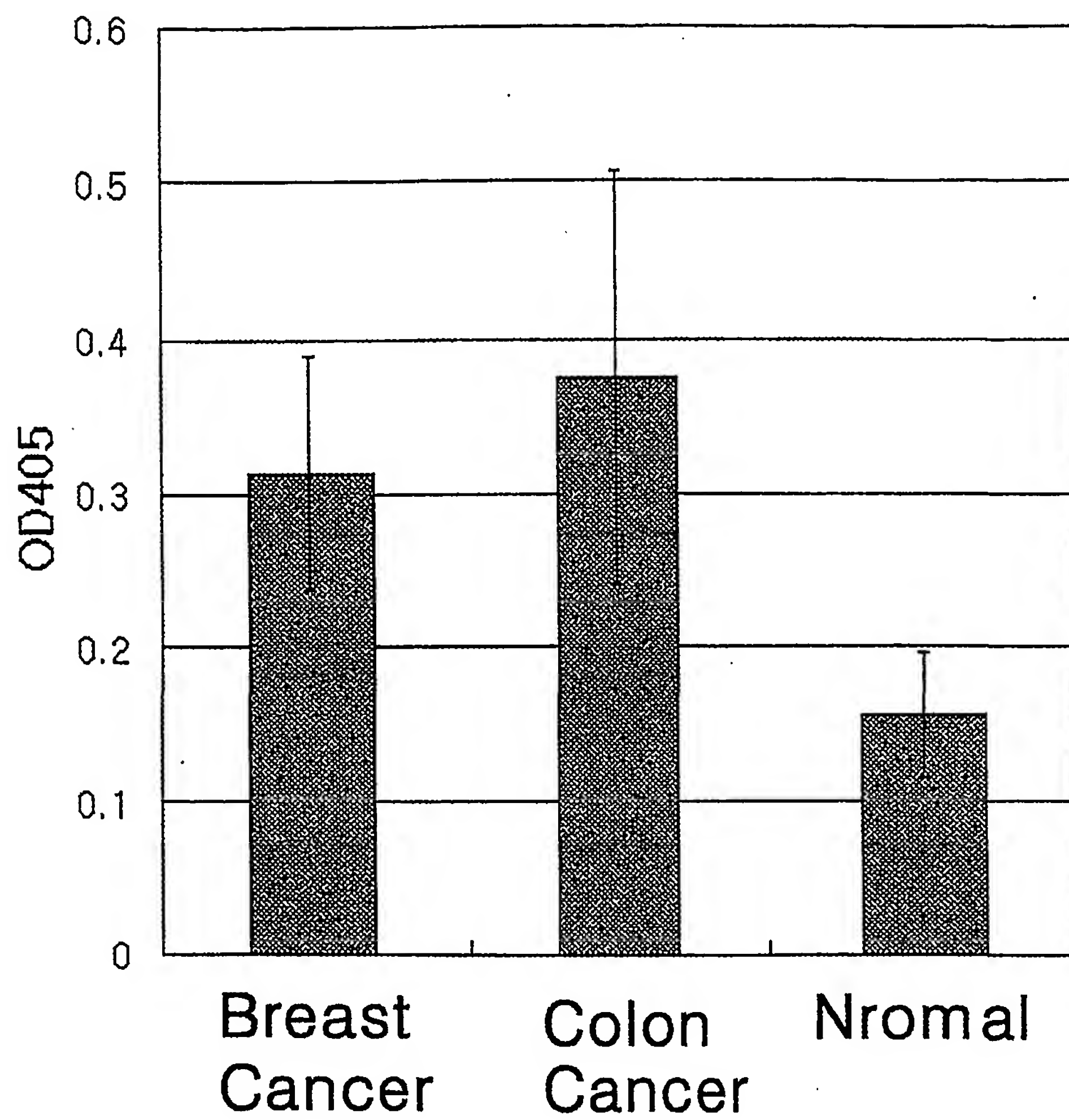
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**FIG. 9**

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**FIG. 10**

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**FIG. 11**

# Sequence Listing

<110> RNA CO., LTD.

<120> Novel stress protein with chaperone activity

<160> 14

<170> KopatentIn 1.71

<210> 1

<211> 730

<212> PRT

<213> Chinook Salmon Embryos

<400> 1

Met Ala Ala Gly Glu Pro Val Asp Val Asn Gly Asp Gly Thr Val Trp  
1 5 10 15

Glu Ile Thr Leu Arg Thr Asp Leu Lys Phe Gln Asp Gly Thr Pro Ile  
20 25 30

Asn Ala Asn Thr Phe Asp Tyr Ser Trp Arg Gln Leu Leu Asp Pro Asp  
35 40 45

Gln Leu Asn Val Arg Ala Ser Asn Leu Cys Asp Ser Ser Asn Leu Pro  
50 55 60

Leu Val Asn Ala Glu Ala Tyr Thr Lys Gln Lys Ser Gln Lys Ser Asp  
65 70 75 80

Asp Phe Asn Glu Pro Ile Trp Asp Ala Gly Asp Lys Gly Thr Ile Glu  
85 90 95

Thr Ala Asn Ala Phe Ala Phe Glu Asp Asp Gly Asp Asp Phe Phe His  
100 105 110

Ala Asn Val Asp Gly Tyr Phe Val Tyr Tyr Tyr Pro Ser Glu Ala Gly  
115 120 125

Thr Phe Gly Gly Asn Trp Asp Leu Leu Tyr Gly Lys Asp Gly Gly Gly  
130 135 140



# Sequence Listing

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Tyr Gly Gly Trp Val Leu Gln Asp Ala Asp Gly Val Pro Thr Val Leu  
 145                      150                      155                      160

Pro Asp Gly Thr Pro Asn Pro Ala Ala Gly Glu Lys Ala Lys Tyr Tyr  
                     165                      170                      175

Gln Gly Ala Lys Pro Lys Leu Phe Tyr Glu Asp Gly Thr Val Val Pro  
                     180                      185                      190

Val Gly Glu Lys Gly Gln Pro Val Gly Ala Thr Ala Ile Pro Asn Asp  
                     195                      200                      205

Pro Val Ser Trp Asp Lys Val Gly Phe Lys Val Thr Gly Thr His Lys  
                     210                      215                      220

Phe Gln Ile Thr Leu Thr Glu Pro Lys Asp Ala Trp Tyr Val Lys Gly  
                     225                      230                      235                      240

Gln Leu Met Ser Gly Ile Thr Gly Val Val His Glu Ala Lys Tyr Glu  
                     245                      250                      255

Ala Gly Lys Ser Ala Asp Lys Arg Thr Thr Thr Tyr Gly Thr Ile Asp  
                     260                      265                      270

Asn Pro Leu Val Ser Tyr Gly Ala Tyr Asn Leu Ile Glu Trp Gln Asp  
                     275                      280                      285

Gly Val Leu Tyr Leu Phe Glu Arg Asn Asp Lys His Tyr Ala Ala Glu  
                     290                      295                      300

Glu Tyr Arg Ile Lys Arg Ile Arg Tyr Asp Val Ile Ala Asp Gln Ser  
                     305                      310                      315                      320

Ile Ala Val Asn Glu Phe Lys Ala Gly Arg Leu Asp Ile Val Gly Ala  
                     325                      330                      335

Ser Gly Glu Tyr Phe Lys Glu Phe Lys Glu Ser Pro Gln Leu Lys Leu  
                     340                      345                      350

# Sequence Listing

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Thr Pro Ser Thr Val Phe Phe Arg Phe Ala Phe Asn Ile Asp Glu Arg  
 355 360 365

Pro Asp Gly Thr Thr Asn Pro Ile Leu Gly Tyr Lys Glu Phe Arg Gln  
 370 375 380

Ala Phe Tyr Tyr Ala Ile Asp Arg Glu Glu Phe Ala Ser Glu Val Arg  
 385 390 395 400

Ala Pro Ala Tyr Ala Asn Gln Gly Phe Ile Gly Pro Val Tyr Ile Ala  
 405 410 415

Thr Glu Tyr Gly Ser Val Ser Tyr Arg Gly Ser Asp Ala Gly Lys Ala  
 420 425 430

Val Phe Ala Pro Leu Ser Pro Glu Thr Thr Gly Phe Asn Pro Thr Lys  
 435 440 445

Ala Lys Gln Leu Phe Asp Ala Ala Tyr Ala Lys Ala Val Ala Asp Asn  
 450 455 460

Lys Ile Ser Asp Gly Gln Lys Val Ser Val Glu Tyr Thr Tyr Tyr Lys  
 465 470 475 480

Val Glu Thr Asn Asp Thr Val Ala Asp Trp Val Lys Gln Thr Val Glu  
 485 490 495

Ala Ile Phe Gly Asp Lys Phe Glu Leu Lys Leu Asn Gly Val Ser Ser  
 500 505 510

Asp Ala Leu Asp Ala Ala Trp Asp Asn Phe Asp Phe Asp Met Thr Phe  
 515 520 525

Gly Gly Trp Gln Gly Leu Asn Phe Asn Pro Val Ser Leu Met Gly Gln  
 530 535 540

Val Tyr Asn Ser Ile Ser Gly Ala Ala Asn Met Leu Glu Asn Gly Phe  
 545 550 555 560

Asp Thr Gly Asn Ile Gln Leu Ser Ile Glu Leu Pro His Thr Lys Ala

# Sequence Listing

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565

570

575

Ala Leu Gln Lys Trp Val Ala Glu Phe Asn Glu Glu Thr Ala Ser Glu

580

585

590

Ala Gln Leu Ala Ala Tyr Gly Arg Trp Thr Ala Ala Leu Ala Lys Phe

595

600

605

Gly Asp Ser Asn Thr Tyr Ser Asp Thr Leu Asp Asn Val Tyr Lys Ala

610

615

620

Ala Ala Ser Thr Phe Tyr Asn Val Ser Asp Val Asn Tyr Ala Gly Arg

625

630

635

640

Asn Asp Asp Phe Asp Arg Ile Thr Ala Ala Phe Glu Ala Ala Leu Leu

645

650

655

Asp Glu Met Met Gln Phe Ser Gly Ala Ser Leu Leu Ser Asp Leu Cys

660

665

670

Ser Gly Ala Gly Ser Arg Ser Gly Leu Pro Ser Val Leu Leu Trp Val

675

680

685

Gly Gln Thr Leu Thr Ser Leu Leu Leu Cys Leu Gly Ala Gly Val Arg

690

695

700

Trp Ala Thr Cys Leu Glu Gly Gly Cys Leu Gly Gly Gly Leu Thr Pro

705

710

715

720

Asp Pro Gly Leu Leu Asp Gly Tyr Asn Asp

725

730

&lt;210&gt; 2

&lt;211&gt; 2744

&lt;212&gt; DNA

&lt;213&gt; Chinook Salmon Embryos

&lt;400&gt; 2

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# Sequence Listing

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acgattgggc taaagcaatt gaagaaggca ctgctactgc agtaggcgac ttacaaaatg	240
cagcatcatt accatatgca cgtgttccaa gaatggcagc tggggaacca gtagacgta	300
atggagacgg tactgtttgg gaaatcacac ttagaactga cctgaagttc caagatggt	360
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taaacgtaag agcaagtaac ctttgcgatt catctaactt accgttagta aacgcagagg	480
cttacacaaa acaaaagagt caaaaagtg atgattttta tgaaccaatt tgggatgcag	540
gtgataaggg aactattgaa acagcaaagc cttttgcatt cgaagatgat ggagatgatt	600
tcttccatgc gaatgtagat ggctattttg tatattacta tccttctgaa gcaggtaactt	660
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cagaacctaa agacgcttgg tacgttaagg gtcaattaat gagtgggtatt acagggtgtg	1020
ttcatgaagc taaatatgaa gctggtaaata cagctgacaa acgtactaca acttacggta	1080
caattgacaa tccattagta tcatatggtg cttataactt aatcgaatgg caagatggcg	1140
tgttatatct attcgaaaga aatgataaac actatgcagc agaagaatat agaattaaga	1200
gaattagata tgatgttatt gctgaccaa gtattgcagt taacgaattt aaagcagggc	1260

# Sequence Listing

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atggaacaac taatccaatt ttaggttata aagaatttag acaagcartc tactatgcaa	1440
ttgaccgtga agaatttgca tctgaagtta gagctcggc ttatgctaac caaggattca	1500
tcggacctgt ctatattgca actgaatatg gttccgtgtc atatcgtgga tcagacgcag	1560
gtaaagcagt atttgctcca ttatcaccag aaacaactgg atttaatcca actaaggcta	1620
aacaactatt tgatgcagct tatgctaagg cagtggctga taataaaatc tcagatggtc	1680
agaaagtatc tgtagaatat acatattaca aagttgaaac taacgatact gttgctgact	1740
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tatcatctga tgcattagac gctgcttggg ataactttga cttcgacatg acatttggtg	1860
gatggcaagg tctaaacttc aatccagttt ccttaatggg tcaagtttat aactcaatta	1920
gtggtgctgc taacatgtta gaaaatggtt ttgatactgg taatattcaa ttaagtatcg	1980
aattaccaca tacaaaagct gctctacaaa aatggggtgc tgaatttaat gaagaaacag	2040
catcagaagc acaattagca gcatatggaa gatggacagc agctttagct aagtttggtg	2100
attcaaacac atattctgac acattagata atgtctacaa agcggctgca tctacattct	2160
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# Sequence Listing

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tggggattat ttggttttaa aactgttctg tttgtttaga tctggttggc tgctgaagtg 2580

ttgctgtgct ggtggagttt gaagctctcc tgttttacac ctgcaagacc tcttgcactc 2640

ctgattgggc agcaaggaag tgggggaaac caggccaagt caattcaacc ctgtgactgt 2700

tctgatgtgt aataaaccta caaattaaaa aaaaaaaaaa aaaa 2744

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<211> 24

<212> DNA

<213> Artificial Sequence

<220>

<223> Degenerate oligonucleotide primer

<400> 3

acnaasgasa cngtngcnga saas 24

<210> 4

<211> 24

<212> DNA

<213> Artificial Sequence

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<223> Degenerate oligonucleotide primer

<400> 4

rttrtcngcn acngtrtcrt tngt 24

<210> 5

<211> 21

# Sequence Listing

<212> DNA

<213> Artificial Sequence

<220>

<223> Degenerate oligonucleotide primer

<400> 5

gargcnaart asgargcngg n

21

<210> 6

<211> 21

<212> DNA

<213> Artificial Sequence

<220>

<223> Degenerate oligonucleotide primer

<400> 6

nccngcster tasttngcst c

21

<210> 7

<211> 26

<212> DNA

<213> Artificial Sequence

<220>

<223> Degenerate oligonucleotide primer

<400> 7

gasgcnggna argcngtntt sgcnc

26

<210> 8

<211> 26

<212> DNA

# Sequence Listing

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<223> Degenerate oligonucleotide primer

<400> 8

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<210> 9

<211> 186

<212> DNA

<213> Artificial Sequence

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<223> oligonucleotide primer

<400> 9

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aaggctaaac aactatttga tgcagcttat gctaaggcag tggctgataa taaaatctca

120

gatggtcaga aagtatctgt agaatacata tattacaaag ttgaaactaa cgatactgtt

180

gctgac

186

<210> 10

<211> 25

<212> DNA

<213> Artificial Sequence

<220>

<223> primer for 5'RACE

<400> 10

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25

# Sequence Listing

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<211> 36  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> anchor primer for 5'RACE

<400> 11  
ggccacgcgt cgactagtac gggnnngggnn gggnnng 36

<210> 12  
<211> 25  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> primer for 5'RACE

<400> 12  
aaattcgta actgcaatac tttagg 25

<210> 13  
<211> 25  
<212> DNA  
<213> Artificial Sequence  
  
<220>  
<223> primer for 5'RACE

<400> 13  
ttcgaataga tataacacgc catct 25

# Sequence Listing

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<210> 14

<211> 36

<212> DNA

<213> Artificial sequence

<220>

<223> universal amplification primer for 5'RACE

<400> 14

ctuactuact uactuaggcc acgcgtcgac tagtac

36



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR01/02139

**A. CLASSIFICATION OF SUBJECT MATTER****IPC7 C07K 14/46**

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC7 C07K 14/46

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and applications for inventions since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Medline, PAJ, Genbank

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ICHIRO et al. 'The 90-kDa stress protein, Hsp90, is a novel molecular chaperone' In:Annals of the New York Academy of Sciences, 1998, Vol.851, p54-60	1 - 80
A	MAYER et al. 'Molecular chaperones: the busy life of HSP90' In:Curr Biol, 1999, Vol.9, no.9, R322-5	1 - 80
A	US 5646249 A1 (KAYE et al.) 08 JULY 1997	1 - 80



Further documents are listed in the continuation of Box C.



See patent family annex.

**\* Special categories of cited documents:****"A"** document defining the general state of the art which is not considered to be of particular relevance**"E"** earlier application or patent but published on or after the international filing date**"L"** document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)**"O"** document referring to an oral disclosure, use, exhibition or other means**"P"** document published prior to the international filing date but later than the priority date claimed**"T"** later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention**"X"** document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone**"Y"** document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art**"&"** document member of the same patent family

Date of the actual completion of the international search

29 APRIL 2002 (29.04.2002)

Date of mailing of the international search report

29 APRIL 2002 (29.04.2002)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office  
920 Dunsan-dong, Seo-gu, Daejeon 302-701,  
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

HAN, Hyun Sook

Telephone No. 82-42-481-5596



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR01/02139

**Box I** Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to part of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Search Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

I: Claims 1-20, 21-24, 25-27, 62, 28, 29, 30, 45-48, 56-59, 76, 79, 80, drawn to inventions related to protein NSP, process for producing protein NSP, nucleic acid molecules encoding protein NSP, etc.

II. Claims 25-27, 60, 61, 62, 63, 69-75, 78, drawn to nucleic acid molecules encoding protein NSP, DNA vaccine containing the said nucleic acid, and nucleic acid array.

III. Claims 31-33, 34, 35, 36, 41-43, 44, 64-68, 77, drawn to antibodies against protein NSP, hybridoma producing the said antibody, and pharmaceutical composition containing the antibody, array of the antibodies.

IV, Claims 37-40, 49-55 drawn to inhibitors of activity or expression of protein NSP and pharmaceutical compositions containing the inhibitors.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be established without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

information on patent family members

International application No.

PCT/KR01/02139

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 5646249 A1	08.07.97	none	